THE UNIVERSITY OF ALBERTA

STUDIES ON PROCARYOTIC SUPERHELICAL DNA RELAXING PROTEINS

by

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A THESIS

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ABSTRACT

Proteins capable of relaxing superhelical DNA have been isolated from E. coli, B. megaterium and M. lysodeikticus. The proteins have been purified to different extents; the best preparations of the E. coli protein were essentially homogeneous. Results of studies conducted with the three proteins suggest that as a class they differ in important features from proteins displaying similar activities which have been characterized in a number of eucaryotic cell types. The three procaryotic superhelical DNA relaxing proteins appear to be generally similar to one another in most respects, although the degree of relaxation of negative supercoils varies. The most interesting distinguishing property separating these proteins and their counterparts in eucaryotic cells was their apparent specificity for negative supercoils. None of the procaryotic proteins was able to release positive superhelical turns. The in vivo function of these proteins, and the possible significance of their differing specificities remains unclear.

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LIST OF ABBREVIATIONS

AH	after heating
BH	before heating
BSA	bovine serum albumin
ccc (DNA)	covalently closed circular
mt DNA	mitochondrial DNA
N-C	nicking-closing
oc (DNA)	open circular
pCMB	p-chloromercuribenzoate
pHMB	p-hydroxymercuribenzoate
PEG	polyethylene glycol
RFI (DNA)	replicative form I, ccc duplex
RFII (DNA)	replicative form II, oc
SDS	sodium dodecyl sulfate
sh DNA	superhelical DNA
S	Svedberg
NEM	N-ethylmaleimide
EDTA	ethylenediaminetetraacetic acid
Tris	tris(hydroxymethyl)aminomethane

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CHAPTER I

INTRODUCTION

It has been known for many years that a variety of circular DNAs exist in organisms ranging from the simplest viruses to the most advanced of eucaryotic species (Helinski and Clewell, 1971). The circular nature of the E. coli chromosome, for instance, was first visualized in the autoradiographic experiments of Cairns, more than ten years ago (Cairns, 1963). Among other DNAs now known to be circular are the genomes and replicative intermediate forms (RF's) of a number of bacteriophages (e.g. ϕX174, PM2) and animal viruses (e.g. polyoma, SV40), bacterial plasmid DNAs and the mitochondrial DNAs (mt DNA) of eucaryotes.

Circular DNAs are of several distinct types which vary in their physical properties as well as in their in vivo functions (Bauer and Vinograd, 1974). These include single stranded circles; open duplex circles (oc), containing an endonucleolytic scission ("nick") in one or both strands; covalently closed circular (ccc) duplex DNAs, where both strands are unbroken; as well as circles of multiples of unit length and catenated, or interlocked circles, resembling links of a chain. This discussion will focus on the two monomeric duplex circular forms, which will for convenience be referred to as oc and ccc, and the interrelationship between them.

The ccc state imposes on duplex DNA circles certain topological restrictions which are reflected in altered physical and biological properties. Exploitation of these physical differences has led to the

introduction of a number of assay methods and preparative techniques which have become extremely useful in the characterization of these DNAs and of proteins that interact with them.

A. Superhelical DNA (sh DNA)

All ccc duplex DNAs which have been isolated to date have been shown to be supercoiled in a negative sense *in vitro* (Helinski and Clewell, 1971; Bauer and Vinograd, 1974). This supercoiling arises because these molecules contain a deficient number of hydrogen bonded Watson-Crick duplex turns (i.e. less than one turn per ten base pairs) at the time of covalent closure *in vivo*. (It remains to be demonstrated that any of these DNAs is, in fact, superhelical *in vivo*.) A possible mechanism for the origin of negative supercoiling postulates that a ligand, (for example, a single-stranded DNA binding protein), is associated with the DNA when the last nick is sealed to produce a ccc molecule (Fig. 1). At the moment of closure a quantity known as the topological winding number (α) of the molecule is fixed, and must remain invariant as long as both strands of the duplex remain covalently closed. (Quantitatively, α is equal to the number of revolutions made by one strand of the DNA duplex about the duplex axis when the molecule is constrained to lie in a plane.) If the ligand is subsequently removed, duplex DNA can reform in the previously single-stranded region of the molecule only if a number of negative superhelical turns equal to the number of newly formed duplex turns are introduced in order to maintain the constant value of the topological winding number, α. This concept can be most simply stated by the equation

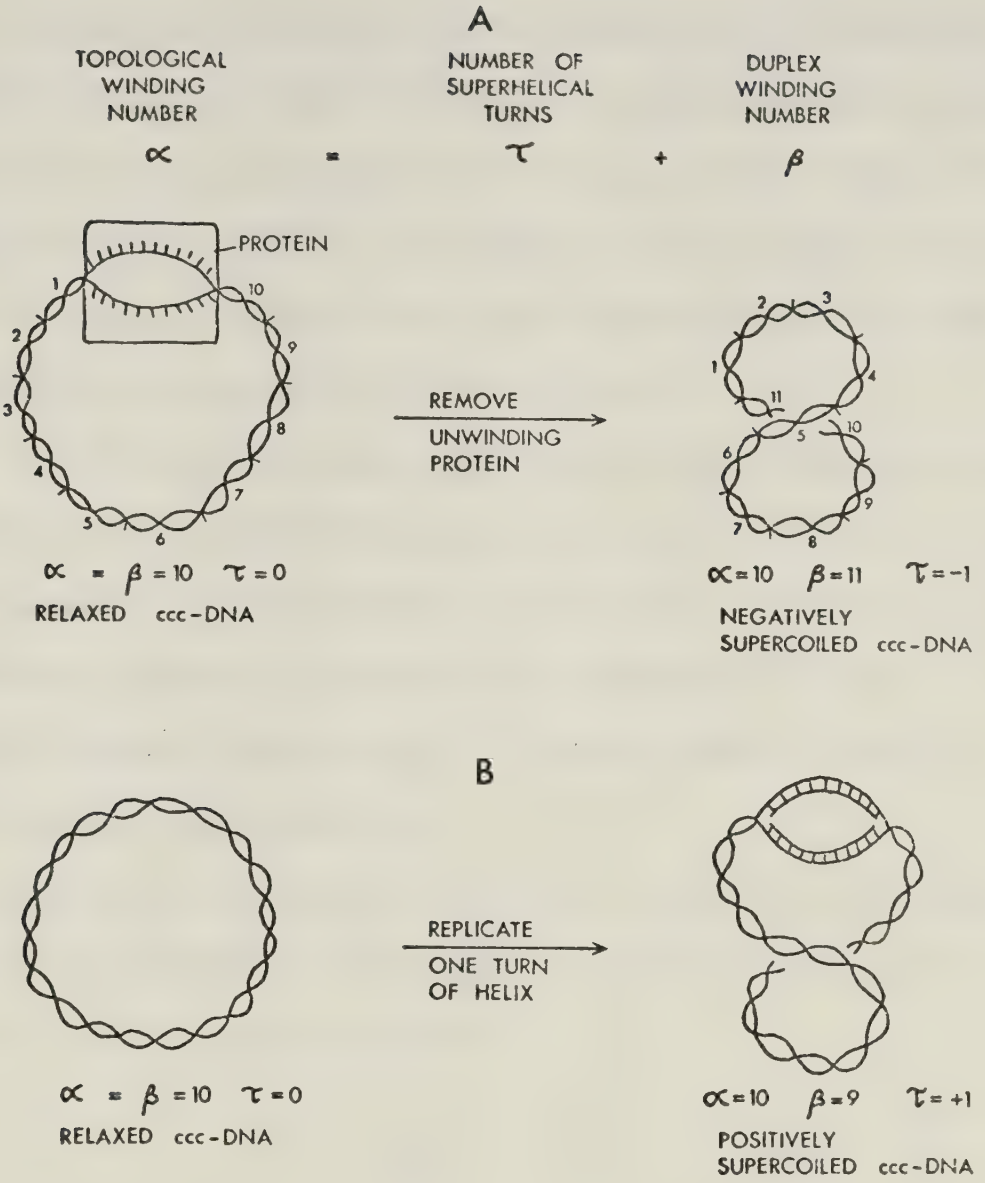

Figure 1 A. A proposed mechanism for the introduction of negative supercoils into ccc DNA in vivo. The DNA is covalently sealed with less than 1 helical turn per 10 base pairs due to the presence of a DNA binding ligand, in this case an unwinding protein. Removal of the ligand allows base pairs to form in the previously unpaired region of the DNA, with the simultaneous formation of 1 negative superhelical turn for each (positive) helical turn.

B. The generation of positive superhelical turns during replication of a ccc duplex DNA molecule. One positive turn is produced for each 10 base pairs unwound as replication proceeds.

$$\alpha = \tau + \beta \qquad (1)$$

originally formulated by Vinograd et al. (Bauer and Vinograd, 1974, and references therein), where β is the duplex winding number, defined as the number of times one strand winds about the duplex axis in the unconstrained molecule, and τ is the number of superhelical turns (i.e. the number of times the duplex winds about the superhelix axis when the superhelix is constrained to lie in a plane).

It must be noted that any agent which affects the winding angle between base pairs in a DNA duplex in such a way as to alter the number of base pairs per turn of the primary helix must, since α is a constant, be reflected in a change in the number of superhelical turns in a ccc DNA. For this reason, superhelical DNAs can be used as sensitive probes of changes in DNA structure.

Another commonly described property of ccc DNAs is the superhelix density (σ), defined as the number of (positive or negative) superhelical turns per ten base pairs:

$$\sigma = \frac{\tau}{\beta^\circ} \qquad (2)$$

where β° is equal to 1/10 the number of base pairs the DNA would have in the B form. Equation (1) can be rewritten as:

$$A = B + \sigma \qquad (3)$$

where A is the topological winding density, $A = \frac{\alpha}{\beta^\circ}$ and B is the duplex winding density, $B = \frac{\beta}{\beta^\circ}$.

The topological constraint on a ccc duplex DNA is reflected in an altered free energy of formation, which is a function of the superhelix density of the DNA. The potential for interconversion of duplex and superhelical turns, subject to the requirement that α remains

constant, means that the most stable structure for a ccc DNA is not determined by helix winding alone. All naturally occurring DNAs of this type behave as if they were deficient in duplex turns at the moment of closure, and formation of supercoils is preferred to a reduction in helix winding density (Bauer and Vinograd, 1974).

A superhelix is a more ordered structure for DNA, implying a reduction in entropy compared to the oc form. This, together with bending and torsional stresses associated with the altered structure of the duplex leads to the prediction that the superhelical conformation is energetically unfavorable compared to the relaxed form of the same DNA, and a release of energy would be expected when the supercoils are released (for example, by endonucleolytic scission of a phosphodiester bond). Similarly, any chemical reaction which results in a reduction in the number of duplex turns should be energetically favorable in a sh DNA relative to the same reaction carried out on DNA having no (or fewer) negative supercoils, while any reaction tending to increase the (positive or negative) superhelix density would be inhibited. Examples, some of which will be considered in more detail later, include the binding of intercalative drugs, and reaction with formaldehyde. Bauer and Vinograd (1970) calculated from ethidium bromide binding data that the formation of 15* superhelical turns in SV40 DNA requires the expenditure of 100 Kcal/mole.

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*Based on the assumption that the angle of unwinding of the helix (ϕ_e) per bound ethidium molecule was 12°. The currently accepted value for ϕ_e is 24° to 26°, corresponding to approximately 30 superhelical turns in the SV40 DNA. See the following discussion on ethidium and its effects on DNA.

The tertiary winding of the duplex in a sh DNA results in a more compact structure than in the corresponding relaxed molecule. Due to the decreased hydrodynamic volume altered sedimentation and viscometric properties are observed (Bauer and Vinograd, 1974); with the magnitude of the differences depending on the superhelix density of the supercoiled species. For example, polyoma DNA (in 2.83 M CsCl at neutral pH) sediments at 21S if supercoiled and at 16S in the oc form (Bauer and Vinograd, 1974). Wang (1969a) has shown that the sedimentation coefficient of a ccc DNA varies in a complex manner with the superhelix density.

The melting behavior of ccc duplex DNA is altered relative to the homologous nicked form as well, another consequence of the topological restriction on unwinding of the duplex and of the presence of supercoiling. The inherent positive free energy of supercoiling acts as a destabilizing factor and facilitates an "early" melting of the duplex, bringing about a reduction in the negative superhelix density. This thermodynamically favorable early melting will continue until all of the negative supertwists originally present have been released. Further melting of the duplex results in the introduction of positive supercoils, an energetically unfavorable process. Even at the point where the DNA is fully denatured, the two strands are unable to separate, and the original value of α is maintained. The principle here is the same whether duplex unwinding is induced by raising the temperature, by alkali titration, or by the presence of an intercalating drug.

Several techniques are available for following the change in tertiary structure when a ccc duplex DNA is unwound. Vinograd and

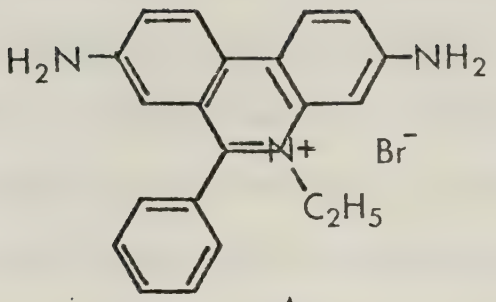
Lebowitz (1966), in their pioneering study of superhelical polyoma DNA, followed the change in sedimentation coefficient in CsCl upon alkali titration. The superhelical form sedimented about 25% faster than the oc DNA until pH 11.4, at which point early melting of the sh DNA began; by pH 11.6 the two forms of the DNA sedimented at the same rate. Thereafter, the S value for the ccc form began to increase again as further titration induced the formation of positive supercoils. Buoyant density shifts have also been employed to monitor changes in the degree of supercoiling. Although buoyant density is insensitive to the shape of DNA at neutral pH, alkali titration leads to Cs^+ salt formation and a consequent increase in density. Therefore, a shift in buoyant density of the ccc relative to the oc DNA began to be observed at the pH (11.4 for polyoma DNA) at which the early melting of the ccc DNA started. (The oc did not change in density until it melted in highly co-operative fashion at pH 11.8.) When the ccc DNA was fully titrated, this difference in buoyant density amounted to 18 mg/ml (Vinograd et al., 1968). At pH values below the onset of early melting, no difference in buoyant density was observed.

Any compound which interacts with a DNA duplex (not necessarily by covalent bonding) in such a way that a change in the winding angle of the duplex takes place will alter the superhelix density of a ccc DNA. If the reaction results in a decrease in the absolute value of σ, the reaction will be energetically favorable since a reduction in the free energy of superhelix formation will result. Conversely, any reaction tending to increase the superhelix density will be unfavorable, and will proceed only as long as the free energy decrease due to ligand binding offsets the increase in superhelix free energy. For any

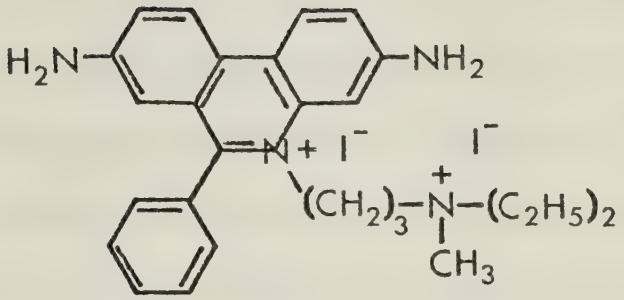
natural, negatively supercoiled DNA, such a ligand will bind preferentially to this form relative to a relaxed DNA at any ligand concentration tending to reduce the number of negative supercoils.

The most widely studied of such compounds have been the intercalating drugs. These compounds, due to their planar polycyclic structures are able to slip between the base pairs of a DNA duplex, causing a stiffening of the helix and a decrease in the winding angle between adjacent base pairs. Figure 2 illustrates the structures of some typical intercalating compounds. The compound in this class upon which the most attention has been lavished is the antitrypanosomal drug ethidium bromide. Much of the early work in this area was performed by Waring, who reported (Fuller and Waring, 1964) that the intercalation of one ethidium molecule caused an unwinding of 12° ($\phi_e = -12°$) in the primary helix. This value was generally accepted and used as a basis for the calculation of the superhelix densities for all of the commonly studied ccc DNAs. More recently, data from two sources (Wang, 1974a; Pulleyblank and Morgan, 1975a) have indicated that a more reasonable value for the unwinding angle is in the range of 24° to 26°, and this has become the currently accepted figure. As a result of these findings, previously reported superhelix densities will need to be revised; in this thesis any figures on superhelix densities given are based on the assumption that $\phi_e = -24°$, unless otherwise noted.

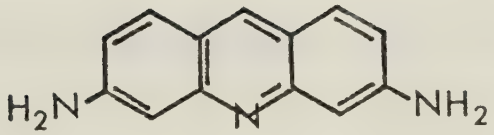
Intercalative binding of ethidium bromide and other related compounds has been exploited in the development of analytical and preparative techniques for the study of superhelical DNAs. At saturating concentrations of the drug, an oc or linear DNA, which



A



B



C

Figure 2 Structures of some typical DNA intercalating compounds. Note the polycyclic aromatic ring system of each compound.
a). ethidium bromide b). propidium diiodide
c). proflavine

lack any topological limitations on its capability to unwind, will be able to bind more ethidium, (to a maximum of one molecule per two nucleotide pairs (Waring, 1965)) than will a ccc DNA (Bauer and Vinograd, 1968). Because ethidium is less dense than Cs DNA, there will be a shift to a lower buoyant density by whichever form of the DNA binds more ethidium. At low concentrations of dye, where unwinding results in loss of negative supercoils, the ccc DNA will bind more ethidium than the oc, and will band at lower density in CsCl. At very high dye concentration where the topological restriction inhibits further binding to the now positively supercoiled ccc DNA, the oc form can still take up dye to the saturating ratio of 0.25 molecules per nucleotide, and will now band at lower density than the ccc DNA. For example, the RFI and RFII forms of SV40 DNA differed in buoyant density by 40 mg/ml in a CsCl gradient in the presence of 100 μg of ethidium per ml (Watson et al., 1971). In the absence of the drug, there was no measurable difference in buoyant density at neutral pH. Dye-buoyant density centrifugation is now routinely used in the purification of superhelical DNAs.

Change in sedimentation velocity with ethidium concentration is another useful parameter in the characterization of ccc DNAs. At low concentration of drug, a decrease in sedimentation coefficient of ccc DNA is observed as drug binding causes a decrease in superhelix density, until at a drug concentration just sufficient to remove all supercoils, oc and ccc forms of the same DNA sediment at the same rate. At higher concentrations of ethidium, further unwinding introduces positive supercoils and the sedimentation coefficient of the ccc DNA again increased (Gray et al., 1971). Both this sedimentation velocity

dye titration method, and the buoyant density-dye titration technique have been employed in studies designed to calculate the superhelix densities of various ccc DNAs (Bauer and Vinograd, 1968, 1974).

LePecq and Paoletti reported (1967) that the binding of ethidium to duplex DNA was accompanied by a 25-fold enhancement in the measured fluorescence. Considerable use has subsequently been made of this observation to develop assays for various structural features of DNA, for DNA synthetic and degradative enzymes, and for proteins whose actions affect the structure of DNA (Paoletti et al., 1971; Morgan and Paetkau, 1972; Coulter et al., 1974; Pulleyblank and Morgan, 1974).

The assay generally employed for sh DNA takes advantage of the preferential binding of the drug to supercoiled DNA relative to relaxed DNA at low drug concentration. At any concentration of ethidium less than that required to completely relax negative supertwists, more of the drug will intercalate into the supercoiled than into the relaxed DNA, yielding a greater fluorescence enhancement for a given quantity of DNA. If the assay is carried out at a pH near 12, a short heating of the DNA samples to about 95° results in the irreversible denaturation of any oc or linear DNA present. Since short intrastrand duplex segments do not form at this pH, there is no return of the fluorescence of this DNA upon cooling. ccc DNA, however, is unable to separate into single strands when heated, and rapidly renatures when cooled with 100% return of the observed fluorescence (Morgan and Pullyblank, 1974). These fluorescence techniques form the basis for most of the sh DNA relaxation assays which will be described in this thesis.

Another technique which has become extremely useful in the study of sh DNA is agarose gel electrophoresis. Based on the obser-

vation that superhelical polyoma or SV40 DNA exhibits a greater mobility than either linear or oc DNA (Thorne, 1967; Sharp et al., 1973), Keller and Wendel (1974) refined the technique to show that it is possible to separate ccc DNAs on the basis of differences in their superhelix densities. This method has been widely employed in assays for sh DNA relaxing enzymes (Keller and Wendel, 1974), in the determination of superhelix densities (Keller, 1975b; Espejo and Lebowitz, 1976; Shure and Vinograd, 1976; DeLeys and Jackson, 1976a), as well as to demonstrate the heterogeneity of σ values in a population of ccc DNA molecules (Dépew and Wang, 1975; Pulleyblank et al., 1975; DeLeys and Jackson, 1976b).

The ability of sh DNA to preferentially bind ligands whose interaction brings about a decrease in superhelix density would be expected to apply to proteins as well as to intercalating drugs. A number of proteins involved in reactions with DNA have been examined with regard to the effect of supercoiling of the DNA on their binding affinities, the most exhaustively studied being E. coli RNA polymerase. Hayashi and Hayashi (1971) studied the template activities of the RFI and RFII forms of ϕX174 DNA in an in vitro assay system. It was shown that the rate of transcription from the superhelical template was three to four times higher than that from the oc DNA, and that this difference was due to an increase in the stability of the initiation complexes formed between RNA polymerase and the supercoiled DNA. This was attributed to preferential binding of RNA polymerase to non base paired regions thought to exist within the sh DNA (Dean and Lebowitz, 1971).

Botchan et al. (1973) extended work in this area using a series

of λ DNAs of different superhelix densities prepared by ligating oc DNA molecules in the presence of various concentrations of ethidium and then removing the ethidium by extraction with butanol (Wang, 1971a). Their results indicated that RNA synthesis increased with increasing τ from 0 to -220, but began to decrease for DNA with τ = -320. However, transcription from the nonsupercoiled λ DNA was found to most closely resemble *in vivo* RNA synthesis in that early genes were preferentially transcribed and RNA synthesis was inhibited by λ C_I repressor protein. This led to the conclusion that the increased transcription on supercoiled templates resulted from initiation at promoter sites, outside the early gene region, which are infrequently used *in vivo*. The authors suggested that there could exist a significant role *in vivo* for structural alterations which affect DNA winding (and, therefore, supercoiling). Two possible explanations for the results were advanced: first, that RNA polymerase may unwind the helix as a prelude to initiation, this would be favored due to the concomitant reduction in the free energy of superhelix formation; or, secondly, that the DNA must assume a new conformation at the promoter site before RNA polymerase is able to bind, and that this conformational change involves unwinding of the duplex.

Richardson (1974) performed similar experiments using superhelical, oc and relaxed ccc DNAs from phage PM2 as templates. He reported that the superhelical DNA was six times as effective a template as oc or relaxed ccc for *E. coli* RNA polymerase. Similarly to the results of Botchan *et al.* (1973), no significant differences were seen in the rates of RNA chain growth on the various templates; the increase in RNA synthesis being due to a greater frequency of

initiation of RNA strands on the superhelical template. Different conditions of ionic strength and divalent metal ion concentration were required for maximum rates of RNA synthesis, and synthesis on the superhelical template was less sensitive to inhibition by ethidium bromide. The explanation for the relationship of transcription rate to supercoiling was believed to lie in structural differences between the superhelical and relaxed forms of DNA template, and could be due either to the presence of high affinity single strand regions on the sh DNA (as suggested by Hayashi and Hayashi, 1971), or to the energetically favorable release of supertwists when RNA polymerase binds to the template (Saucier and Wang, 1972).

Wang (1974b) examined the effect of changing the superhelix density of PM2 DNA on the catalytic properties of the core and holoenzyme forms of E. coli RNA polymerase, E. coli DNA polymerase I, pancreatic DNase and the single-strand specific endonucleases from N. crassa and mung bean. It was found, as predicted from previous work, that the ability of the DNA to act as a template for RNA synthesis increased with superhelix density up to $\sigma = -0.08$ and began to decrease at higher superhelix densities. Results were the same whether the core or holoenzyme form of RNA polymerase was used. The sensitivity of the DNA to pancreatic DNase was independent of the extent of supercoiling at all available values of σ. The rate of initial cleavage of the DNA by single stranded endonucleases showed a dependence on superhelix density only when $-\sigma > 0.08$, suggesting that no significant increase in single strandedness was necessary in producing lower degrees of superhelicity. None of the supercoiled molecules was able to act as a template for DNA synthesis catalyzed by DNA polymerase I, even in the

presence of short oligonucleotides added as primers. On the basis of these results, Wang has questioned the interpretation of Dean and Lebowitz (1971) that supercoiled ϕX174 and PM2 DNAs would contain about 4% single stranded regions, and suggested that their results could be explained in terms of an early melting by reaction of supercoiled DNA with formaldehyde or methyl mercuric hydroxide, similar to that observed by Vinograd et al. (1968) for alkali titration of superhelical polyoma DNA.

In another related study, Wang (1974c) measured the dependence of lac repressor binding to λp lac DNAs of differing superhelicities. The results indicated that negative supertwists stabilized the repressor-operator complexes in DNAs of up to $\tau = -320$, and concluded that the binding of one repressor molecule tended to unwind the DNA helix by approximately 80°. At values of $-\tau > 160$, the affinity of repressor for the DNA began to decrease, similar to previous observations on the effect of supercoiling on RNA synthesis.

Vogel and Singer (1975, 1976) studied the interaction of histone H1 with SV40 DNA at different values of τ by a nitrocellulose filter binding assay. It was shown that binding to filters was at a minimum when $\tau = 0$ and increased with increasing τ in either a positive or negative sense. These data require a different interpretation than has been advanced for the binding of other proteins, since the free energy of superhelix formation would tend to inhibit the binding to positively supercoiled DNA of any ligand which unwinds the primary helix. It was suggested that the H1 histone may be recognizing some structural feature of the supercoils themselves in an unknown manner. This may be explainable in regard to the proposed in vivo function of

H1, which does not appear to be directly involved in nucleosome formation in chromatin (Kornberg, 1974; Kornberg and Thomas, 1974), but is thought to be involved in the folding of internucleosomal DNA (Bradbury et al., 1974).

Champoux and McConaughy (1975) investigated the template properties of superhelical SV40 DNA with $\tau = -116$. Their data showed that a fraction of the nascent RNA, ranging in size from 80 to 600 nucleotides, was associated with the superhelical template in a form which was resistant to RNase in high salt, and thermal melting experiments confirmed that the RNA was hydrogen bonded to the DNA. When relaxed ccc SV40 DNA was used as template, this RNA-DNA complex was not found. The complex could be purified and was able to serve as template-primer for DNA synthesis catalyzed by E. coli DNA polymerase I; a covalent linkage of DNA to the RNA primer was demonstrated. The ccc template-primer was able to support only limited synthesis of DNA. A slow rate of endonucleolytic nicking of the template was observed over the time course of the reaction, and the nicked template was able to support considerably greater synthesis of DNA, suggesting that the topological constraint in the ccc complex prevented extension of the nascent DNA strand beyond a certain length. It appeared that the nick, by providing a swivel point for the relaxation of the topological block, allowed continuation of DNA synthesis; alternatively, the nick could be simply providing a new 3'OH terminus from which a new DNA strand could be initiated. No single or double stranded endonuclease activity was detectable in the DNA polymerase preparation used and the authors did not determine the mechanism by which the nicks were introduced. No mention was made of any experiments to test the effects of

addition of eucaryotic sh DNA relaxing protein on the rate and extent of DNA synthesis from the ccc template-primer.

Richardson (1975) further investigated the problems of initiation of RNA synthesis and association of nascent RNA with native supercoiled PM2 DNA (τ = -110); unlike Champoux and McConaughy (1975), who used SV40 DNA with a superhelix density about 2.5 times greater than the naturally-occurring species. Richardson found that with either superhelical or relaxed ccc DNA, nascent RNA was hydrogen bonded to the template by less than 20 nucleotides. When a protein denaturing agent such as SDS or phenol was added, all RNA was released from complexes with the relaxed DNA, while 60% of the newly synthesized RNA was retained by the supercoiled template. Moreover, this 60% was now more tightly bound to the template, up to 600 nucleotides being RNase resistant, corresponding to an unwinding of 60 turns of primary DNA helix and a release of 60 of the 110 negative superhelical turns originally present.

The susceptibility of supercoiled and relaxed ccc ϕX174 DNA to cleavage by N. crassa single stranded endonulcease after fixation by hydroxymethylation with formaldehyde has been investigated by Kato et al. (1973). It was found that the superhelical form could be fixed at 22°, while a temperature of about 42° was required to fix the relaxed ccc DNA to the same extent, suggesting that the sh DNA contains a region that is either weakly hydrogen bonded or unpaired. There was only one such region detectable per ϕX circle, present due to the strain inherent in superhelix formation. A more recent study (Bartok and Denhardt, 1976) indicated that the DNA could be cleaved in either strand and that there was not a unique cleavage site. These data are

consistent with the earlier studies of Dean and Lebowitz (1971) on the possibility of single-stranded regions in superhelical DNA, an interpretation which has been challenged by Wang (1974b).

Holloman et al. (1975) have shown that ϕX174 RFI is able to incorporate single-stranded fragments of homologous ϕXDNA to a maximum of 90 nucleotides per molecule. The incorporated DNA was resistant to exonuclease I, and not readily displaced by heating to 60° unless a nick had been introduced into the circle. Relaxed circular DNA was not able to bind the nucleotide fragments, and the uptake by superhelical DNA was inhibited by ethidium bromide (50% inhibition occurred at a concentration of ethidium of 0.43 μg/ml, 1.1 μM, calculated as sufficient to release 9 of the 41 negative supertwists in the DNA). Addition of ethidium to complexes released the bound fragments, with 50% release at 0.66 mM, indicating a competition for binding to the superhelical DNA. Transfection experiments using complexes formed from am3 ϕXDNA and fragments of wild-type DNA showed that the fragments could be incorporated into am+ recombinant progeny phage. The experiments support the suggestion that the uptake of a portion of a single strand of DNA by a duplex could be a crucial event in the initiation of recombination (Meselson and Radding, 1975; Benbow et al., 1975).

B. Proteins Which Relax Superhelical DNA

The first activity capable of altering the topological winding number, α, of a ccc DNA was detected in a cell free extract of E. coli 1100 by Wang (1969b). Subsequently, this activity was purified about one thousand fold, shown to be a protein, and designated omega (Wang, 1971b). Using a sedimentation velocity assay method, Wang showed that

omega protein was capable of removing most of the negative superhelical turns from ccc DNA of λb2b5c, leaving a product which was still covalently closed in both strands. The product did not differ significantly in any physical property measured from a DNA of similar superhelix density which had not been treated with omega. The reaction was shown to require a divalent cation but no other cofactor, and to follow multiple-hit kinetics. Circular DNA which had been forced into a positively supercoiled conformation by the addition to the reaction mixture of ethidium bromide was not a substrate for omega protein. It was postulated that omega acted by reversibly introducing a swivel into the phosphodiester backbone of the DNA and that the reaction mechanism involved a phosphoprotein intermediate. Another important and distinguishing feature of the activity of this protein was that the product DNA retained some residual negative superhelical turns. More recently, revised purification and assay procedures were described (Wang, 1973; Carlson and Wang, 1974), and the protein was shown to have a molecular weight of between 100,000 and 110,000 in one subunit. Temperature sensitive mutants in DNA replication were assayed for omega activity; none of the dna A to G mutants examined exhibited a thermosensitive omega. No tests were carried out on transcriptional ts mutants.

A eucaryotic protein having a similar activity to E. coli omega was reported by Champoux and Dulbecco (1972). This superhelix relaxing activity was isolated from the nuclei of secondary mouse embryo cells and was assayed by its effect on the buoyant density of radiolabelled polyoma sh DNA in CsCl propidium diiodide gradients. These workers showed that the eucaryotic protein was unable to seal nicks in

oc DNA and had no conventional ss DNA binding (DNA unwinding) activity. Interestingly, the protein was active on both positively and negatively supercoiled DNA substrates, and reduced the superhelix density to zero. Since the preparation was not highly purified, the authors were unable to state whether the activity was catalytic or stoichiometric. It could not be ruled out that the protein existed in a "charged" form and would require recharging after once acting on a substrate DNA molecule. A possible *in vivo* role as a swivel in DNA replication was suggested for the protein; the introduction of positive supertwists ahead of the replication fork would be expected if the parental DNA was a ccc duplex (Jaenisch *et al.*, 1971). A similar mechanism to that proposed by Wang (1971b) was envisioned for the eucaryotic protein, which was given the designation "DNA untwisting enzyme".

In 1974, Baase and Wang reported the isolation of an omega protein from the fertilized eggs of *Drosophila melanogaster*. This protein resembled the mouse embryo protein in its features: no physical change other than the removal of superhelical turns could be detected in the product DNA, the reaction followed multiple-hit kinetics and no cofactor was required for activity. As well, the *Drosophila* omega was able to relax both native, negatively supercoiled DNA and DNA with ethidium-induced positive supercoils. Optimal activity required 0.2 M KCl, but no Mg^{++} or other divalent cation, and complete relaxation was seen. The protein exhibited no DNA ligase activity. The activity was reported to exist primarily in the cytoplasm, nuclei having been separated from the crude homogenate by centrifugation. However, an apparently identical activity was obtained from purified nuclei, suggesting a lack of compartmentalization of the activity.

Heating for 5 minutes at 50°C or treatment with 0.03 mM SDS resulted in inactivation of the protein, with no separation of the endonucleolytic and ligating functions. The postulated nicked DNA-protein intermediate could not be detected.

Sen and Levine (1974) found that when SV40 infected African Green Monkey kidney cells were lysed in the presence of Triton X-100 and 0.2 M NaCl (to preserve viral DNA as a rapidly sedimenting nucleoprotein complex) a sh DNA untwisting activity was found associated with the viral DNA. The activity was shown, by a CsCl/ethidium bromide density gradient assay, to relax exogenous supercoiled SV40 DNA. Untwisting activity was found in complexes with both mature viral and replicative intermediate (RI) DNA and was absent from uninfected cells lysed in the same manner.

Vosberg et al. (1975) demonstrated the presence of similar activities (which they called "relaxation proteins") in the nuclei of mouse LA9 and HeLa cells. Unlike the omega protein from Drosophila eggs (Baase and Wang, 1974), the protein from these cells showed very little activity in the cytoplasm (2% of the nuclear level) or mitochondria (< 1%). In their properties these proteins resembled previously-studied eucaryotic omega proteins (Champoux and Dulbecco, 1972; Baase and Wang, 1974), being active on both positively and negatively supercoiled DNA substrates, and requiring 0.2 M NaCl. Using both buoyant density-dye and fluorescence enhancement assay methods, the activity was purified to the point where a single band of molecular weight 37,000 was observed on SDS-polyacrylamide gels. The purification procedure resulted in a 25-fold increase in specific activity and approximately 1% yield. The activity was inhibited 70% by 1 mM pHMB, suggesting

the presence of an essential sulfhydryl group. Activity could also be inhibited by denatured or (less effectively) by native DNA, but not by tRNA. No separation of the nicking and resealing functions was observed and the postulated nicked intermediate was not seen.

Keller (1975a), using the agarose gel assay technique described above, purified a protein which he called "DNA-relaxing enzyme" from human KB cells, achieving a 1000-fold purification with 6% yield. A series of discrete DNA bands of intermediate mobility observed on the agarose gels indicated that reduction of the number of superhelical turns proceeded in a stepwise fashion, in agreement with Wang (1971b) and Champoux and Dulbecco (1972). The activity was sensitive to pronase, SDS, and heat and was inhibited by 0.3 mM NEM or 0.3 mM pCMB. The molecular weight obtained by sedimentation in sucrose was 70,000; and by SDS-polyacrylamide gel electrophoresis was 60,000 ± 3,000. Since the sedimentation coefficient for the native enzyme was greater than predicted for a spherical molecule of this molecular weight, it was suggested that the active form could consist of two subunits of molecular weight 60,000. In other respects the KB cells protein was found to be similar to other eucaryotic sh DNA relaxing proteins; it relaxed positive as well as negative supercoils, and was optimally active in the presence of 0.2 M NaCl, with 50% activity remaining if 10 mM $MgCl_2$ was substituted. It was calculated, assuming a molecular weight of 60,000, that one molecule of the protein was capable of relaxing a minimum of 10 SV40 sh DNA molecules, and therefore could properly be referred to as an enzyme. As in previous work, no successful isolation of a nicked DNA intermediate was achieved.

Pulleyblank and Morgan (1975b) reported the partial purification

of an omega protein from calf thymus and described a simple, rapid assay method based on the fluorescence enhancement of ethidium bromide when it binds to DNA by intercalation. Relaxation of negative supercoils caused a fluorescence drop, while positive relaxation resulted in an increase in fluorescence for a given quantity of sh DNA. The calf thymus omega activity was associated with chromatin, from which it could be extracted by washing with high salt buffers. Two omega-rich fractions, one eluting at 0.7 M NaCl, and the second at 1.7 M NaCl, were seen. The properties of the two fractions, designated ω^1 and ω^2, were very similar, and it was suggested that the reason for their appearance at different salt concentrations could be that the omega bound to different histone fractions in the chromatin preparation. The calf thymus omega(s) resembled the other previously described eucaryotic sh DNA relaxing proteins in catalytic properties. Attempts to trap a nicked phosphoprotein intermediate with hydroxylamine were unsuccessful.

A rapid procedure for the purification of a sh DNA relaxing protein from mouse L cell nuclei has recently been reported (Vosberg and Vinograd, 1976). The protein eluted from a Sephadex gel column at a position expected for a globular protein of molecular weight 75,000; on SDS-polyacrylamide gels a single band of 35,000 was seen. The ability of a given amount of the protein to relax a 20-fold molar excess of supercoiled PM2 DNA indicated that the activity was enzymatic. Agarose gel and CsCl-ethidium density gradient centrifugation experiments indicated that an enzyme molecule would preferentially relax one sh DNA molecule completely before releasing and attaching to another DNA. The 12% yield of protein in the most

purified fraction was calculated to correspond to between 1% and 2% of the total nuclear protein. "Nicking-closing (or N.-C.) enzyme" was advanced as a suitable name for proteins of this type.

Pulleyblank et al. (1975) found that the limit product of a nicking-closing reaction carried out over a long period of time (from 24 to 48 hours) in the presence of a 20-fold excess of enzyme was not a topologically homogeneous species. Using a modified agarose gel system which separated negatively from positively supercoiled species having the same absolute value of τ, they showed that the product could be resolved as a series of bands with a mean degree of supercoiling of $\tau = 0$ and that the relative masses of the bands (quantitated by densitometric tracings of photographic negatives of the stained gels) followed a Boltzmann distribution. Furthermore, treatment of relaxed ccc DNA with N.-C. enzyme generated a similar distribution of product species, indicating for the first time that a nonsupercoiled DNA could be a substrate for the enzyme. Any single band purified from a gel and incubated a second time with the enzyme was resolved into the entire set of bands when electrophoresed again. These results were explained on the basis of random rotation about the swivel point due to thermal fluctuation during the period when the DNA molecules were transiently nicked.

Treatment of oc DNA with polynucleotide ligase produced a product which could be resolved into an identical series of bands on agarose gels. This observation was confirmed by Depew and Wang (1975), who further showed that the position of the centre of distribution of bands varied with the temperature at which the ligase reaction was performed, due to the temperature dependence of the helix rotation

angle (Wang, 1969a).

A recent communication from Wang's laboratory (Depew et al., 1976) indicated that further progress had been made in the study of E. coli omega protein. This report showed that under carefully defined conditions a partial omega-DNA reaction could be induced and that covalent protein-DNA complexes could be isolated. This marked the first successful demonstration of the existence of the intermediate species which had been generally postulated by investigators in this field (Fig. 3). It was found that treatment of the complex with alkali or detergent caused the introduction of a nick, with the protein remaining attached to the 5' end of the nicked strand.

Since mitochondrial DNA is circular, the possibility existed that mitochondria could be a source of sh DNA relaxing protein. Fairfield et al. (1976), employing a procedure designed to minimize the chances of contamination by a nuclear activity, discovered and partially purified a mitochondrial N.-C. enzyme. The protein was found, by electron microscopy, to completely relax negative supercoils in PM2 DNA; activity in positively supercoiled DNA was not reported.

To date, no clear idea of the in vivo role of sh DNA relaxing proteins is available. It is apparent, however, that any cellular process which is affected by the degree of supercoiling of chromatin (in eucaryotes) or "free" DNA (in procaryotes) could, potentially, be subject to regulation by the activity of a protein of this type. The probability of sh DNA relaxing proteins playing a role in DNA replication must be considered. Observations that transcription by E. coli RNA polymerase, binding to DNA by lac repressor, and the interaction of histone f1 with DNA, are all influenced by the degree of supercoiling

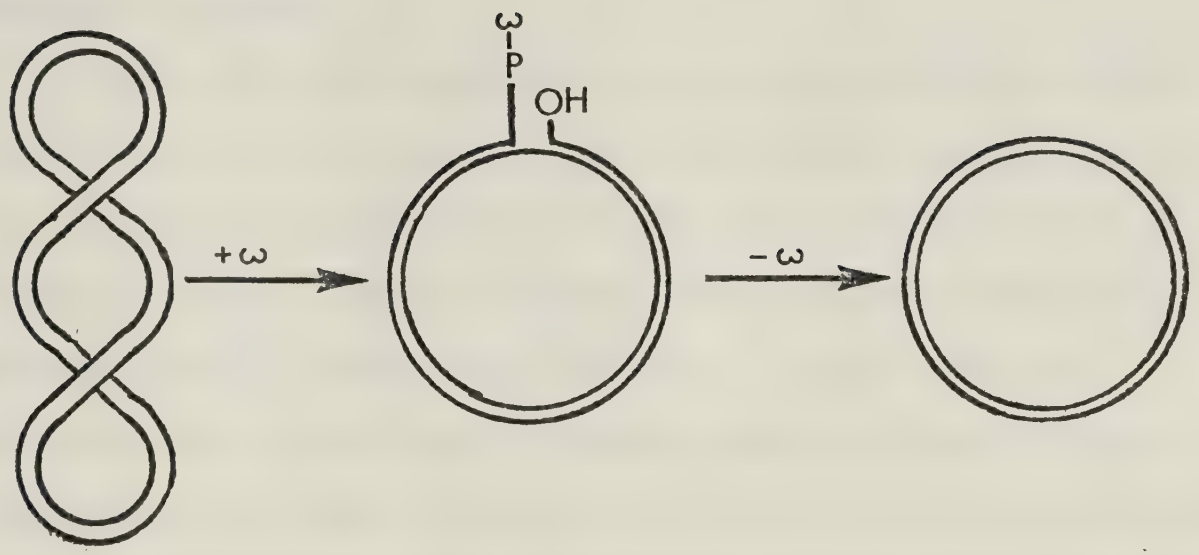


Figure 3 The proposed mechanism for the relaxation of negative supercoils by a sh DNA relaxing protein (ω). The important feature of the mechanism is the preservation of the energy of the cleaved phosphodiester bond in a phosphoprotein intermediate. The illustration shows the removal of 2 negative supertwists, reducing the superhelix density to 0. In practice, supercoils are removed singly, with the nick being resealed each time.

of the DNA have already been discussed. It has also been shown that sh DNA will take up homologous ss DNA fragments by a mechanism suggestive of events related to the initiation of recombination. Thus, although solid evidence is lacking as yet, it is clear that the range of possible functions for these proteins is wide. It furthermore seems reasonable to believe that sh DNA relaxing activities will prove to be ubiquitous in nature.

One of the more intriguing aspects of the story of sh DNA relaxing proteins is the apparent differences between E. coli omega, the only procaryotic protein of this type for which published data is available, and the eucaryotic proteins which have been studied by numerous groups of investigators (Table 1). In all cases where sufficient data has been made available, eucaryotic sh DNA relaxing proteins have been shown to be active on both positively and negatively supercoiled DNA, reducing the superhelix density to approximately zero and preferring a reaction medium containing 0.2 M NaCl or KCl. The E. coli omega, on the other hand, is apparently specific for negative supercoils, does not yield a completely relaxed product, and is inactive in the presence of 0.2 M salt.

The availability of a convenient, rapid fluorometric assay for sh DNA relaxing activity was an important consideration in the decision to carry out further studies on E. coli omega protein and, it was hoped, similar proteins from other procaryotes. The possibility of isolating and characterizing mutants deficient in this activity, with a view to elucidating an in vivo function, was also explored, but is not a major aspect of the studies to be reported. For the most part, this thesis will deal with the partial purification of omega-like

TABLE 1.

Properties of sh DNA Relaxing Proteins Previously Reported

Source	Mol. Wt.	Subunits	Relaxes: +ve	Relaxes: -ve	Salt Requirement	Reference
E. coli	100K-110K	1	-	+	2 mM Mg^{++}	Wang (1969b, 1973)
mouse embryo	N.D.	N.D.	+	+	0.2 M NaCl	Champoux and Dulbecco (1972)
Drosophila eggs	N.D.	N.D.	+	+	0.2 M NaCl	Wang (1974)
SV40/AGMK	N.D.	N.D.	N.D.	+	0.1 M NaCl	Sen and Levine (1974)
mouse LA9	75,000	2 X 35,000	+	+	0.2 M NaCl	Vosberg and Vinograd (1974) Vosberg *et al.* (1975)
human KB	120,000 or 60,000	1 or 2 X 60,000	+	+	0.2 M NaCl or 10 mM MgCl*	Keller (1975a)
calf thymus	N.D.	N.D.	+	+	0.2 M NaCl	Morgan and Pulleyblank (1975b)
liver mitochondria	N.D.	N.D.	N.D.	+	N.D.	Fairfield *et al.* (1976)

* approximately 50% active

proteins from other species of bacteria, and a study of some of their properties. The results generally support the conclusion that eucaryotic and procaryotic sh DNA relaxing proteins are fundamentally different.

Terminology has as yet not been finalized in this field - a variety of terms (omega protein, DNA untwisting enzyme, DNA relaxing enzyme, unswivilase, nicking-closing enzyme) has been used by different groups of workers to describe what is essentially the same activity; that is, the nicking of a superhelical DNA molecule, a release of superhelical turns by rotation about the nick, and finally closure by covalent rejoining of the two ends of the nick (Fig. 3). For purposes of this thesis, the term superhelical DNA (or sh DNA) relaxing protein has been adopted as it seems to describe the function of the protein most accurately.

CHAPTER II

MATERIALS AND METHODS

A. Sources

PM2 DNA was purified as previously described (Morgan and Pulleyblank, 1974). Generally, between 80% and 90% was covalently closed circular.

DNA Agarose, prepared by the method of Schaller *et al.* (1972) was 4% agarose and bound approximately 3.5 mg of denatured calf thymus DNA per ml of gel.

DEAE cellulose (DE-23) was purchased from Whatman and was swollen and washed according to the instructions of the supplier.

Electrophoresis grade agarose was supplied by Sigma Chemical Co. Acrylamide and N,N'-methylene bisacrylamide were products of Eastman Kodak Co.

Ethidium bromide was purchased from Sigma; stains-all (1-ethyl-2[3-(1-ethylnaphtho[1,2d]-thiazolin-2-ylidene)-2-methylpropenyl]naphtho [1,2d]-thiazolium Bromide, $C_{30}H_{27}BrN_2S_2$) was a product of Eastman Kodak Co.

Naladixic acid was a gift of Dr. J. Davies.

Other chemicals were reagent or enzyme grade; stock solutions were millipore filtered.

E. coli B, grown to 3/4 log phase, was purchased from Grain Processing Co. as a frozen paste and was stored frozen until used.

Stock cultures of *Bacillus megaterium* and *Micrococcus lysodeikticus* (*M. luteus*) were gifts from Dr. W. Paranchych of this

Department. Small quantities of these cells were grown in sterile trypticase soy broth in a shaking water bath at 37°, and, after harvesting, stored frozen until used. Large-scale growth of B. megaterium was carried out in a 300 l fermentor in half strength trypticase soy broth at 37° with constant aeration. Cells were harvested in late log phase ($O.D._{650}$ = 2.0) in a continuous flow centrifuge and stored frozen.

Protein concentrations were determined by the method of Lowry et al. (1951).

DNA samples for electronmicroscopy were prepared according to the method of Davis et al. (1971), with 40% formamide and 0.1 mg of cytochrome C per ml in the spreading solution. The hypophase contained 10% formamide and DNA was picked up on 150 mesh parlodion coated copper grids. Grids were stained with 5 X 10^{-5} M uranyl acetate and rotary shadowed with 12 to 15 Å of platinum-carbon at an angle of 8°.

B. Assays for sh DNA Relaxing Activity

1. Fluorescence assay.

Fluorescence enhancement was measured using a Turner Model 430 Spectrofluorometer with the cuvette compartment maintained at a temperature of 25° by means of a circulating water bath. Wavelengths of excitation and emission were 525 and 600 nm respectively. The high pH ethidium bromide assay cocktail contained 20 mM potassium phosphate, 0.1 mM EDTA and 0.5 µg/ml ethidium bromide; final pH of the cocktail was 11.8, measured against a standard buffer of pH 12.45.

Generally, samples containing approximately 1 µg of PM2 DNA (20 µl @ $1A_{260}$/ml) were added to 2 ml of the assay cocktail. The

instrument was preset to an arbitrary value of 50 on the X100 scale with a standard which consisted of 10 µl of calf thymus DNA at a concentration of 1 A260/ml in 2 ml of the assay cocktail. The reading of this standard was re-checked periodically through the course of a large number of readings to prevent errors due to instrument drift. The standard was freshly prepared each day. (If aliquots of reaction mixtures were not read soon after being taken, the samples were kept in the dark as a precaution against light induced cleavage of DNA by ethidium (Denniss and Morgan, 1976)). Two ml of assay cocktail which contained no DNA was used as a blank. Separate cuvettes were used for blank, standard and sample readings; these had been previously matched to ±1 fluorescence unit on the X100 scale.

The actual fluorescence measurement was a two-step process. The fluorescence of each sample was measured once, then the sample tubes were covered with parafilm and heated at 95° for three minutes. After cooling the samples to 25° in a rapidly stirring waterbath, the fluorescence of each was measured a second time. At the high pH of the assay cocktail, linear duplex and nicked circular DNA do not renature or form intrastrand duplex regions upon cooling, and therefore show less than 1% return of fluorescence after the heat step (Morgan and Pulleyblank, 1974). Covalently closed circular DNA, however, renatures completely, and 100% return of fluorescence is observed.

A relaxation of negative supercoils was indicated by a fluorescence drop both before heating (BH) and after heating (AH). Using PM2 DNA, a drop in fluorescence of 33% was observed for full relaxation (Pulleyblank and Morgan, 1975b), because relaxation reduces affinity of the DNA for ethidium. Relaxation of positive supercoils permits

increased ethidium binding, and an increase in fluorescence is seen both before and after heating. Endonucleolytic nicking is typified by an increase in fluorescence of up to 30% before heating due to release of the topological constraint on ethidium binding, and a corresponding drop of up to 100% after heating, which denatures the nicked molecules irreversibly.

2. Agarose gel assay

Gels were 1% agarose in 40 mM tris acetate (pH 7.8), 10 mM sodium acetate and 1 mM EDTA, prepared by dissolving agarose in the rapidly stirring buffer at 90° to 95°. The solution was cooled to about 60° before being poured into 15 cm X 0.6 cm (i.d.) tubes. The lower ends of the tubes had been constricted to about half their original diameter by fire-polishing as a means of preventing the gels from slipping out during electrophoresis. Prior to use, the gel was extruded slightly from the top end of the tube and sliced with a razor blade to produce a flat surface onto which the sample could be layered.

Samples were generally prepared by adding 20 µl of reaction mixture (containing DNA at $1A_{260}$/ml) to 5 µl of cold 0.2 M EDTA to quench a Mg^{++} dependent reaction (or 5 µl of 5 M NaCl to quench a Mg^{++} independent reaction). To this were added 2 µl of 0.1% bromophenol blue as marker dye and 3 µl of 40% ficoll or 25λ of 60% glycerol. The bottom end of each gel tube was covered with a piece of dialysis membrane held in place by a thin section of rubber tubing. The electrophoresis buffer was the same as that in which the gels were made up. Generally, the electrophoresis was run for 14 to 18 hours at low voltage (1 to 2 volts/cm), which was sufficient to allow PM2 sh DNA to migrate approximately

3/4 of the length of the gel.

Following electrophoresis the gels were stained for visualization. In early experiments, the stain used was 0.5 µg/ml ethidium bromide in 10 mM tris, 0.1 mM EDTA. Under ultraviolet light of 254 nm the stained bands were a bright orange color; however, it proved impossible to prepare useful photographs with the available equipment. For this reason gels were stained instead with 0.005% Stains-all in 50% formamide. With freshly-prepared stain, a one-hour staining period was sufficient. Following destaining in water, DNA was visible as blue bands, which could be readily photographed under white light. The major drawback to this procedure is that the stain is very light labile and color disappears from both solutions of stain and from stained gels in a matter of hours on exposure to room light. For this reason, stock solutions of stain were kept in brown bottles, and stained gels were stored in the dark.

C. Conditions for sh DNA Relaxation Reactions

In most cases, the reaction mixture had a final volume of 50 µl and contained 10 mM tris-HCl, pH 8, 2 mM $MgCl_2$, 1 mM Na_3EDTA, gelatin at 80 µg/ml (previously heated for at least one hour at 95°) and PM2 DNA at a concentration of $1A_{260}$/ml (80% to 90% ccc). The reaction mixtures were kept on ice until the fraction to be assayed (usually 2 µl or 5 µl) was added. A zero time sample of 20 µl was removed immediately following addition of the enzyme, and pipetted into 2 ml of the high pH assay cocktail. The remainder of the reaction mixture was then incubated, typically at 30° for 30 minutes. To terminate the reaction the mixture was chilled in an ice bath (for 30 sec.)

before a second 20 µl aliquot was removed and processed in the same way as the zero time sample. The 1:100 dilution of the reaction mixture into the pH 11.8 ethidium bromide assay cocktail resulted in essentially instantaneous quenching of the reaction.

When kinetic experiments were performed, the volume of the reaction mixtures was increased to 150 µl and the DNA concentration to 1.2 A_{260}/ml. In this procedure up to nine aliquots of 15 µl each were taken at incubation times of as long as 3 hours. Once samples had been pipetted into the 2 ml of assay cocktail they were stored in the dark until fluorescence measurements were taken.

The procedure for agarose gel assay experiments was identical except that samples from reaction mixtures were quenched by addition to 5 µl of 0.2 M EDTA.

D. SDS-Polyacrylamide Gel Electrophoresis

The method of Laemmli (1967) was used for SDS-polyacrylamide gel electrophoresis. Gels were 10% acrylamide, 0.26% N,N'-methylene bisacrylamide in 50 mM sodium phosphate, pH 7.1 and 0.1% SDS. Protein samples for gels were prepared by adding 10 to 20 µg of protein fraction to a cocktail containing 5% β-mercaptoethanol, 2% SDS, 10% glycerol and 0.002% bromophenol blue in a total volume of 100 µl. Samples were heated at 95° for 5 minutes before being applied to the gels. After application of samples, gels were pre-run for 10 minutes at 3 mA/gel for 10 minutes, then run at 6 mA/gel until the tracking dye had run to approximately 0.5 cm from the bottom of the gel (between two and three hours for 10 cm gels). The gels were stained for 30 to 60 minutes in

0.05% Coomassie Brilliant Blue in methanol:HOAc:water (5:1:5) and destained in a Canalco Electrophoretic Destainer. E. coli RNA polymerase (a gift of Mr. P. d'Obrenan) was run separately as a molecular weight standard. Destained gels were scanned at 600 nm using a Gilford Model 2400 spectrophotometer equipped with a gel scanning accessory.

E. Superhelical DNA Relaxing Assays on Small Quantities of E. coli

In order to screen for mutants defective in sh DNA relaxing activity it was desirable to perfect a procedure which would allow for the rapid testing of large numbers of small colonies. A practicable method was worked out based on that employed by de Lucia and Cairns (1969) in their search for mutants in DNA polymerase I. An inoculating loop of E. coli B culture was streaked across a plate of nutrient agar, which was then incubated overnight at 37°. Four of the smallest colonies (diameter 1 to 2 mm) were picked using a sterile loop and suspended in 100 µl of 10% sucrose, 0.1 M tris, pH 8, 1 mM EDTA. After brief vortex mixing, freshly prepared lysozyme (1 mg/ml) was added to a final concentration of 50 µg/ml. The suspension was left on ice for 30 minutes, then warmed to room temperature, and made 5% in Brij-58 and 50 mM in $MgSO_4$ in a final volume of 160 µl. The contents of the tube were gently mixed until the lysate cleared and were then spun for 10 minutes at 18,000 rpm in a Beckman Microfuge. The supernatant was assayed for sh DNA relaxing activity by the fluorescence assay as described above.

F. Purification of *E. coli* sh DNA Relaxing Protein

Purifications of *E. coli* sh DNA relaxing protein were carried out starting with 100 g of frozen cells and followed the published procedure of Wang (1971) through the stage of DEAE cellulose chromatography. Wang subsequently further purified his protein by two successive phosphocellulose chromatography steps. In our hands, however, unacceptably high losses of activity were encountered when the active fractions from the DEAE cellulose column were passed over phosphocellulose. In an attempt to overcome this problem, chromatography on a column of single-stranded calf thymus DNA agarose was investigated.

1. ss DNA agarose chromatogrpahy.

The peak fractions of activity, which eluted from DEAE cellulose between 0.1 and 0.2 M NaCl, were pooled and dialysed overnight against buffer A (10 mM tris, pH 8, 1 mM EDTA, 1 mM β-mercaptoethanol, 5% glycerol) containing 0.1 M KCl (in later preparations 0.1 M NaCl was substituted to avoid problems of precipitation when the fraction was subjected to SDS gel electrophoresis). The dialysed protein, fraction IV, was applied to the DNA agarose column (volume 10 ml) using a peristaltic pump. Direction of flow through the column was from bottom to top. After application of the sample, the column was washed with several column volumes of buffer A/0.1 M KCl to remove nonadsorbed protein. A linear gradient of 0.1 M to 1.0 M NaCl in buffer A (total volume 100 ml) was then applied to the column, and fractions of 1.25 ml were collected. A constant flow rate of 7.5 ml/hour was maintained as a faster rate caused the column to pack. Less than 1% of the applied

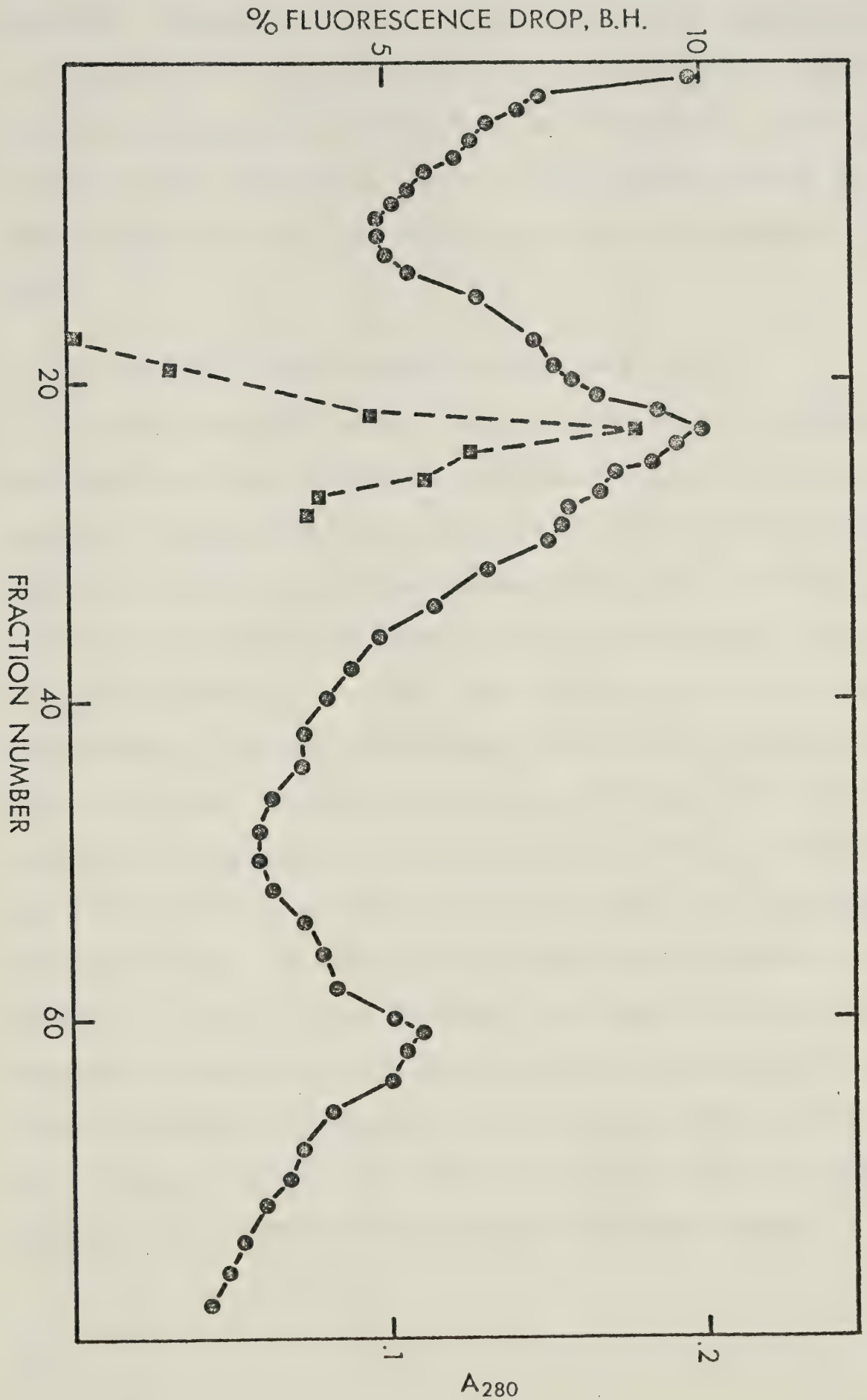
protein was bound to the DNA on the column, and this was eluted in a broad peak (Fig. 4). The bulk of the sh DNA relaxing activity coincided with the absorbance peak; smaller amounts of activity were detectable essentially all the way across the trailing shoulder of the absorbance peak. The three column fractions of highest activity (fractions 22 to 24 in Fig. 4) were combined to give fraction V. Traces of endonuclease were still detectable in the preparation at this stage, indicated by larger fluorescence drops after the heat step than before; a kinetic experiment was performed on column fraction 23 to confirm the presence of sh DNA relaxing protein in this peak (as in Fig. 8). The endonuclease was also widely distributed through the column fractions, no obvious separation of the two activities was observed. The primary virtue of this step is the separation of the active protein from the majority of the protein present in fraction IV, which does not bind to the DNA on the column. Neither sh DNA relaxing activity nor endonuclease was detected in the nonbinding protein. The broad distribution of activity could be an indication of a heterogeneous population of binding sites on the DNA attached to agarose. One possible solution to this problem would be to prepare DNA agarose using a synthetic DNA of defined base sequence, for example, poly d(AT).

When not in use, the DNA agarose column was stored in buffer A containing 0.1 M KCl and 1 mM NaN_3.

2. Gel filtration

Fraction V was concentrated to a final volume of about 2 ml by dialysis against dry sucrose and applied directly to a 35 ml column of Sephadex G100 which had been equilibrated with buffer A in 0.1 M NaCl.

Figure 4 DNA agarose chromatography of fraction IV E. coli sh DNA relaxing protein. Fractions were assayed by adding 5 µl of eluant to standard reaction mixtures (total volume of 50 µl) and measuring the fluorescence drop between 0 and 30 minutes. Samples of 20 µl were removed from the reaction mixture and added to 2 ml of pH 11.8 ethidium assay mixture. Relaxing activity ■----■ , A_{280} ●——●


% FLUORESCENCE DROP, B.H.
5
10
20
40
60
FRACTION NUMBER
.1
.2
A280

The column was eluted with the same buffer, and 0.5 ml fractions were collected. Superhelical DNA relaxing protein eluted from the column in a single peak and was free of detectable endonuclease. Active fractions were pooled to produce fraction VI and stored either at 2° in buffer A/0.1 M NaCl or at -20° in the same buffer plus 50% glycerol. Under either conditions, the activity was stable for a maximum of three weeks.

3. Yield of sh DNA relaxing protein from E. coli.

The yields were somewhat variable in different preparations, but this was due at least in part to the difficulty in quantitating the activity. In the early stages of the purification nucleases interfere with the assay and thus a large increase in apparent activity is seen in going from the ammonium sulfate fractionation (fraction III) to fraction IV (Table 2). As well, the activity was found to be unstable, particularly in its most purified form (fraction VI). Finally, the unit of activity is not accurately measured, and has been defined as the amount of protein just sufficient to produce a 15% drop in the fluorescence of 1 μg of PM2 ccc DNA in 30 minutes at 30° under the standard assay conditions. (An example of the calculations involved is given in Appendix I). However, this required a large number of assays for accurate determination, which did not seem warranted when nuclease contamination existed. The apparent purification is 650-fold from fraction III to fraction VI with a 10% yield. Wang (1971) reported a purification of approximately 1000-fold in his original published method.

TABLE 2.

Purification of sh DNA Relaxing Protein from *E. coli*

Fraction	Volume (ml)	Protein (mg)	Activity (units/mg)	Total Units
III	41	2050	12	24,600
IV	79	450	386	174,000
V	46	11.3	2917	33,000
VI	9.25	2.1	7840	16,500

G. Partial Purification of a sh DNA Relaxing Protein from B. megaterium

All operations were performed at between 2° and 4°; generally 100 g of cells were used for a preparation. The cells were homogenized in a Waring blender with 100 ml of buffer A/0.1 M NaCl until a uniform suspension was achieved. The suspension was sonicated at maximum intensity using a Biosonik III or IV fitted with a large probe. After each one minute burst of sonication, the temperature of the suspension rose to 8° to 10°, and the suspension was therefore cooled back to 2° in an ice-ethanol bath before sonication was continued. Eight to ten one-minute sonications generally disrupted nearly all of the cells.

The crude sonic lysate was centrifuged for 45 minutes at 20,000 xg in a Sorvall RC2-B refrigerated centrifuge to pellet cell debris. The supernatant from this spin was fraction I. Fraction I was made 1.1 M in NaCl, then 40% polyethylene glycol 6000 (PEG) was added, with stirring, to a final concentration of 10%. After the PEG was well mixed, the solution was left in the cold for up to two hours to allow complete precipitation of the DNA. A ten minute centrifugation at 10,000 xg in the GSA rotor was sufficient to pellet the precipitate. The supernatant was fraction II.

Ammonium sulfate was added to fraction II to a final concentration of 20% (W/W) over a period of 20 minutes with constant stirring. After a further 30 minutes' stirring, the solution was centrifuged for 20 minutes at 20,000 xg. This step resulted in a phase separation, with the ammonium sulfate precipitate at the interface between the lower aqueous phase and the upper, PEG-rich, phase. The aqueous phase was removed by suction and sufficient ammonium sulfate was added to

produce a saturated solution. The precipitate was collected by centrifugation as before, dissolved in a minimum volume of buffer A/0.1 M NaCl, and dialysed overnight against the same buffer. The dialysed solution is fraction III.

Fraction III was diluted two-fold with buffer A/0.1 M NaCl and applied to a 10 ml column of single-stranded DNA agarose. The flow rate was maintained at about 7 ml per hour using a peristaltic pump and the direction of flow was from bottom to top of the column. Following application of the sample the column was washed with several volumes of buffer A/0.1 M NaCl and developed with a 0.1 M to 1.0 M NaCl gradient in buffer A. Following the gradient, a wash with 2 M NaCl/buffer A was carried out. Fractions of 1.25 ml were collected from the gradient and subsequent high salt wash.

Column fractions were assayed for sh DNA relaxing activity using the ethidium bromide fluorescence assay method. Activity was found over a very wide range (see Fig. 5). Column fractions 55 to 100 were combined to produce fraction IV, and concentrated by dialysis against dry sucrose. For long term storage the concentrated material was dialysed against buffer A containing 0.1 M NaCl and 50% glycerol. In this state fraction IV could be stored at -20° for at least 6 months without loss of activity, a considerable improvement over E. coli fraction VI.

Superhelical DNA relaxing activity was not detectable in fractions I, II or III due to endonuclease interference. Kinetic experiments (Fig. 6) indicated that small amounts of endonuclease contamination remained in fraction IV. However, the levels were too low to be of serious concern in routine 30 minute assays. Attempts to

Figure 5 DNA agarose chromatography on fraction III *B. megaterium* sh DNA relaxing activity. Details are as in the legend to Fig. 4. Relaxing activity ■---■ , A_{280} ●—●

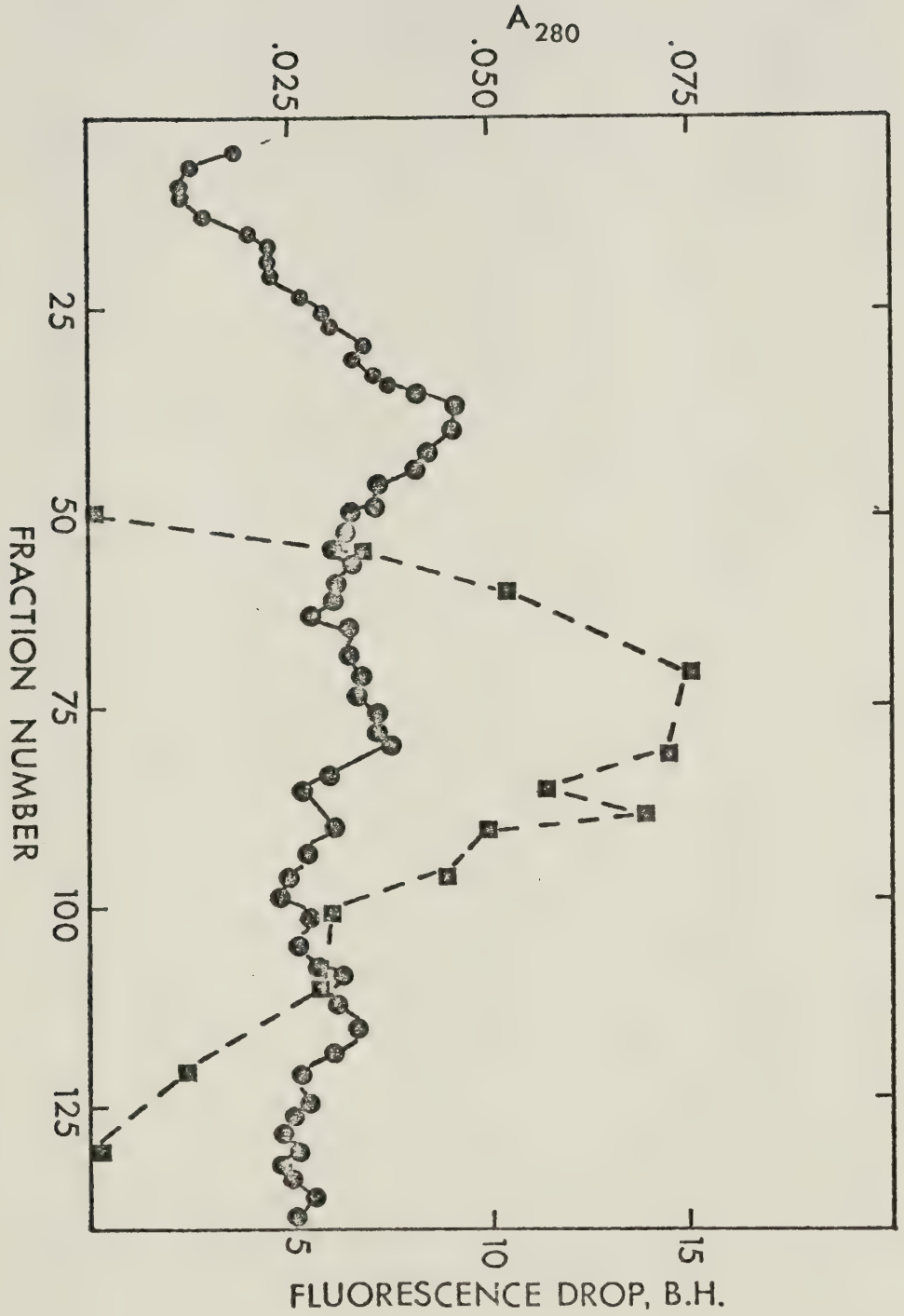
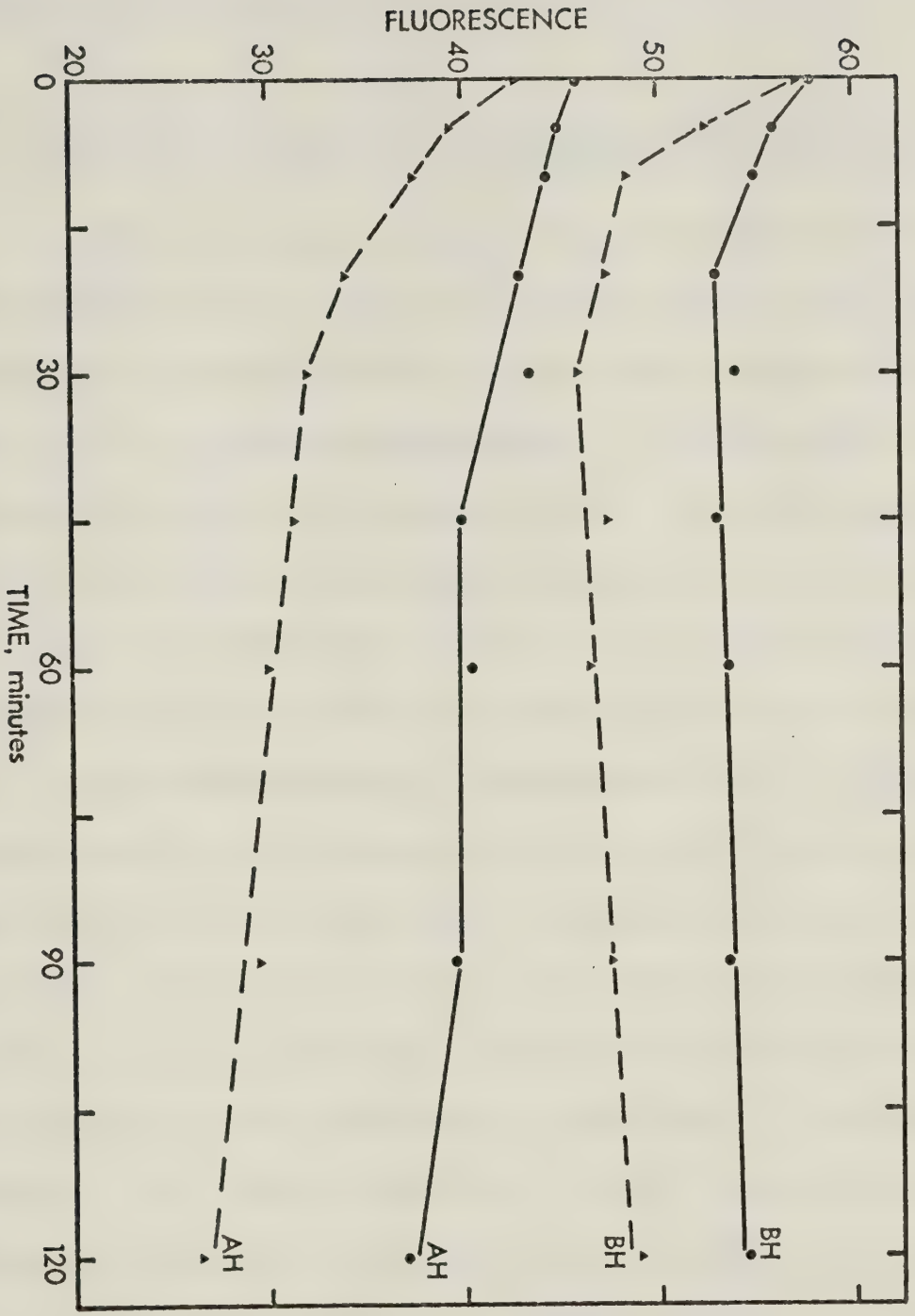

A280
.025
.050
.075
25
50
75
100
125
FRACTION NUMBER
5
10
15
FLUORESCENCE DROP, B.H.

Figure 6 Kinetics of relaxation of PM2 ccc DNA with fraction IV *B. megaterium* sh DNA relaxing activity. Two concentrations of protein were used in 150 µl reaction mixtures. Concentration of PM2 DNA was 1.2 A_{260}/ml; all other conditions were the same as for standard reactions. At each time point 15 µl aliquots were removed and added to 2 ml of pH 11.8 ethidium assay mixture.
●——● 0.6 µg of protein, ▲---▲ 3.0 µg of protein

Fluorescence readings are given in arbitrary units.


FLUORESCENCE
20
30
40
50
60
0
30
60
90
120
TIME, minutes
AH
AH
BH
BH

remove the contamination by chromatography of small amounts of fraction IV on Sephadex G150 were unsuccessful and resulted only in failure to recover the sh DNA relaxing activity, possibly as a result of dilution. Fraction IV contained approximately 300 µg of protein.

H. Proteolytic Cleavage of *B. megaterium* sh DNA Relaxing Protein

Small aliquots of fraction IV were incubated with trypsin at a concentration of 48 µg/ml (i.e. 1:21 dilution of stock solution of trypsin) for varying periods of time at room temperature, then immediately used in sh DNA relaxation assays. A fresh supply of trypsin-treated fraction IV was prepared for each experiment, and the activity of the trypsin was tested before each experiment using a calf thymus DNA-histone H1 (KAP) assay. To 2 ml of a solution containing 10 mM tris-HCl, pH 8, 0.1 mM EDTA and ethidium bromide at 0.5 µg/ml was added 10 µl of calf thymus DNA at a concentration of 1 A_{260} (50 µg/ml). The fluorescence of this solution was set to read 70 on the X100 scale (an equivalent quantity of DNA gives a reading of 50 in the pH 11.8 assay cocktail). To this solution was added sufficient histone H1 (in a volume of 2 µl) to reduce the fluorescence to zero upon mixing. The non-fluorescing histone-DNA was the substrate for the trypsin assay. Usually 5 µl of trypsin (1mg/ml in 1 mM HCl) was added to the solution (final concentration 2.5 µg/ml) and, after mixing, the return of fluorescence was observed as the trypsin digested the histone, allowing ethidium to bind to DNA. If full recovery of fluorescence occurred in 10 minutes or less, the trypsin was considered sufficiently active for use in experiments on fraction IV *B. megaterium* sh DNA relaxing protein.

I. Superhelical DNA Relaxing Activity from M. lysodeikticus

M. lysodeikticus was grown in 15 ml of trypticase soy broth at 37° in a shaking water bath overnight, to a final $O.D._{650}$ of 1.5. Cells were harvested by centrifugation (10 minutes at 10,000 xg in an SS-34 rotor) and resuspended in 0.1 M tris, pH 8.5/10% sucrose (1 ml). To this was added 25 µl of 0.2 M EDTA and 50 µl of freshly prepared lysozyme, 1 mg/ml. The suspension was left on ice for 30 minutes, then warmed to room temperature and made 10% in Brij-58, 0.5% in Na deoxycholate and 50 mM in $MgSO_4$, and mixed very gently for about 5 minutes. The lysate was centrifuged for 40 minutes at 15,000 rpm in an SS-34 rotor, and the supernatant assayed for relaxing activity. The pellet was washed with 1 ml of 1 M NaCl, 10 mM tris, pH 8, 0.1 mM EDTA, and the wash supernatant also assayed. Both supernatants were found to possess relaxing activity by fluorescence assays, with the high salt wash being somewhat richer in activity. Stored at 2° without further treatment, the activity (fraction II) was stable for 2 to 3 weeks.

CHAPTER III

RESULTS

A. *E. coli* sh DNA Relaxing Protein

The molecular weight and subunit structure of *E. coli* sh DNA relaxing protein were determined by SDS-polyacrylamide gel electrophoresis and gel filtration through Sephadex G-100. The SDS gels of fraction V (DNA agarose) and fraction VI (G100) each showed only two bands (Fig. 7A); these corresponded to molecular weights of 56,000 and 31,000 as determined from spectrophotometric scans of stained gels compared with *E. coli* RNA polymerase run as molecular weight standard (Fig. 7B). The endonuclease contamination which was detected in fraction V by the fluorometric assay was not visible as a band on the fraction V gel. This was not surprising, considering the high sensitivity of the nuclease assay (Morgan and Pulleyblank, 1974).

For determination of molecular weight by gel filtration, relaxing protein, *E. coli* DNA polymerase I and bovine serum albumin were run separately on a G100 column. The elution volumes to the A_{280} peak fraction for each protein were: DNA polymerase, 3.5 ml (molecular weight 109,000); BSA, 4.6 ml (molecular weight 69,000) and relaxing protein 4.3 ml. These results are illustrated in Fig. 8. If it is assumed that the sh DNA relaxing protein is a spherical molecule, these data and the SDS gel experiments are consistent with the protein being an αβ dimer having a molecular weight in the region of 87,000. Wang (1973) reported that omega protein, isolated from a different strain of *E. coli*, consisted of a single polypeptide chain having a molecular

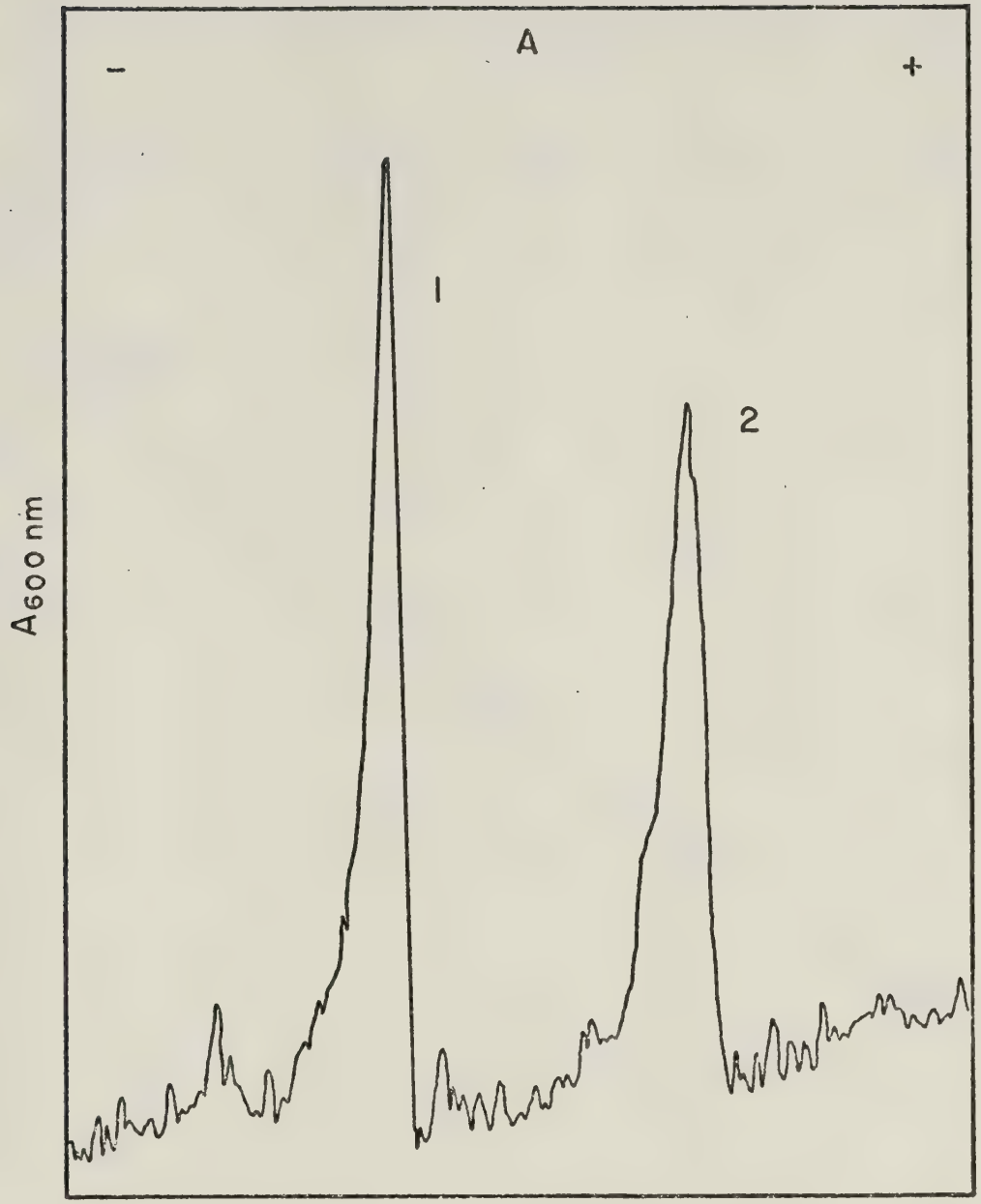


Figure 7 A. Spectrophotometric scan of an SDS-polyacrylamide gel of fraction VI E. coli sh DNA relaxing protein.

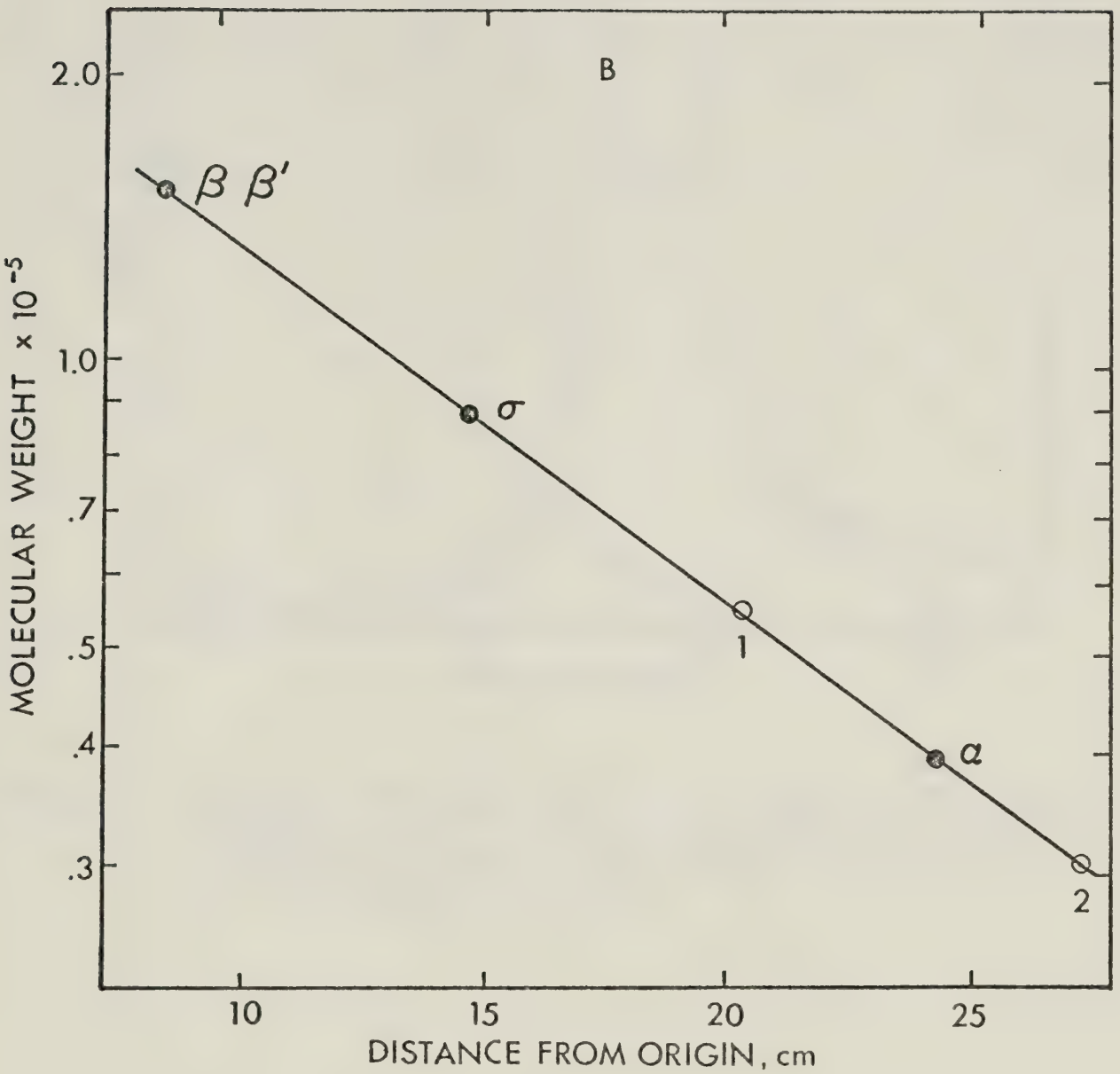


Figure 7 B. Molecular weight determination of the subunits (1,2) E. coli RNA polymerase subunits (β, β', α, σ) as the standards.

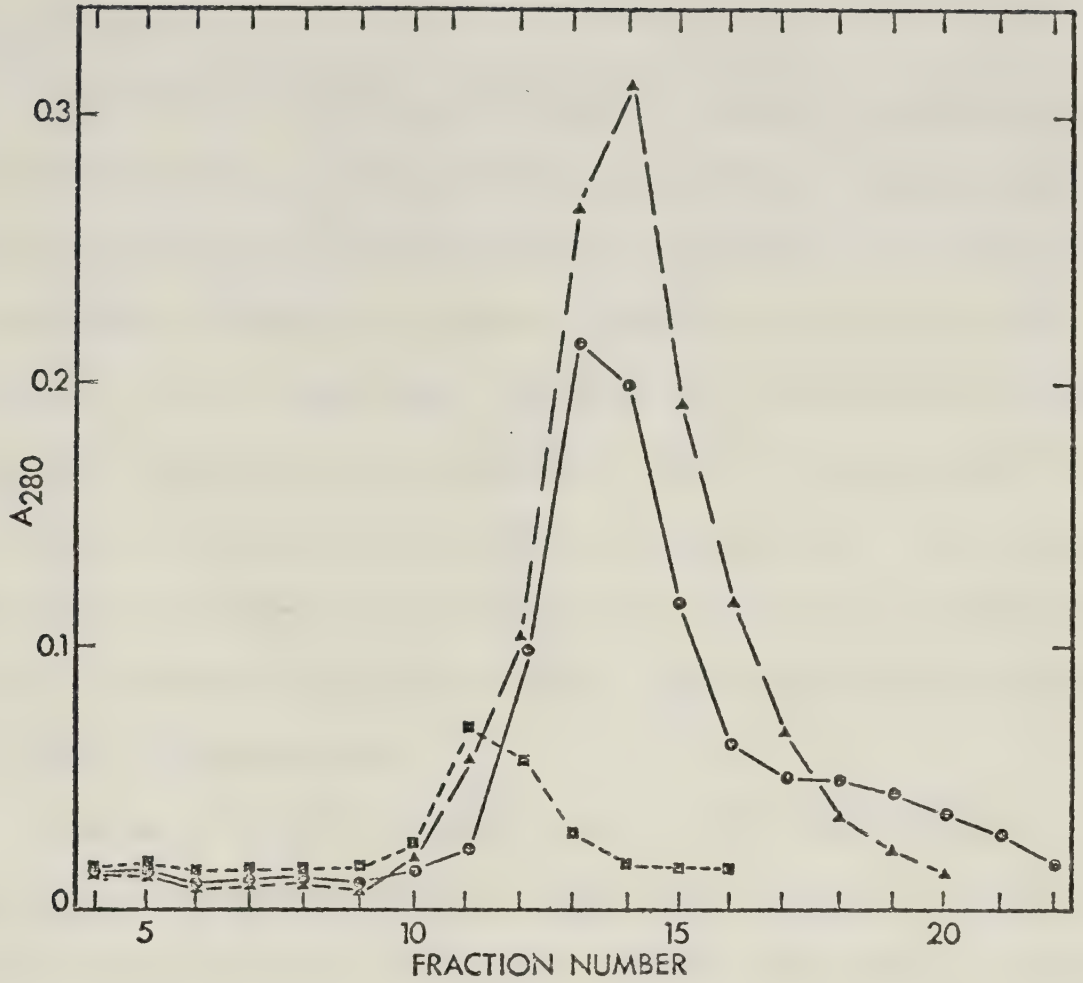


Figure 8 Sephadex G100 chromatography of E. coli DNA polymerase I (109,000 daltons) ■----■ , bovine serum albumin (69,000 daltons) ▲— —▲, and E. coli sh DNA relaxing activity ●——● in buffer A/0.1 M KCl.

weight of 110,000.

That fraction VI was uncontaminated by endonculeases was suggested by kinetic data. Figure 9 shows the results of an experiment tracing the relaxation of PM2 ccc DNA by fraction IV and fraction VI. The initial loss followed by a gradual increase in the before heat fluorescence, and the approximate 50% drop after heat in the fraction IV experiment indicated that a significant level of endonuclease was still present in the protein preparation at this stage. In the case of fraction VI, the fluorescence drops before and after the heat step were nearly identical at all time points, suggesting that the preparation was now free of endonuclease.

Results of studies characterizing the salt requirements of E. coli sh DNA relaxing protein are summarized in Table 3. All the experiments were conducted at 30° for a period of 30 minutes. The data are very similar to the results reported by Wang (1971), notwithstanding the apparent differences in molecular weight and subunit structure. The sh DNA relaxing protein required low levels of Mg^{++} for activity, being fully active up to 4 mM, but completely inactive at 20 mM. This loss of activity at higher levels was probably a salt effect, since KCl at concentrations greater than 50 mM was inhibitory, with no activity remaining at the physiological concentration of 0.2 M. This suggests that, in vivo, the protein either is inactive in the cytoplasm or requires some currently unknown component. Regarding the first possibility, ethanol has been found to stimulate some DNA polymerases (Kornberg and Gefter, 1972), possibly because they are membrane bound; sh DNA relaxing activity, however, was not enhanced and was, in fact inhibited at higher ethanol concentrations. Spermidine concentrations

Figure 9 Kinetics of relaxation of PM2 ccc DNA with fraction IV and fraction VI *E. coli* sh DNA relaxing protein. The standard reaction conditions were used, with the protein fractions being diluted 10-fold into the 150 µl reaction mixtures. Fluorescence change for fraction IV (contaminated with nuclease): ■——■ BH, O—O AH, and for fraction VI ●—● BH and □——□ AH.

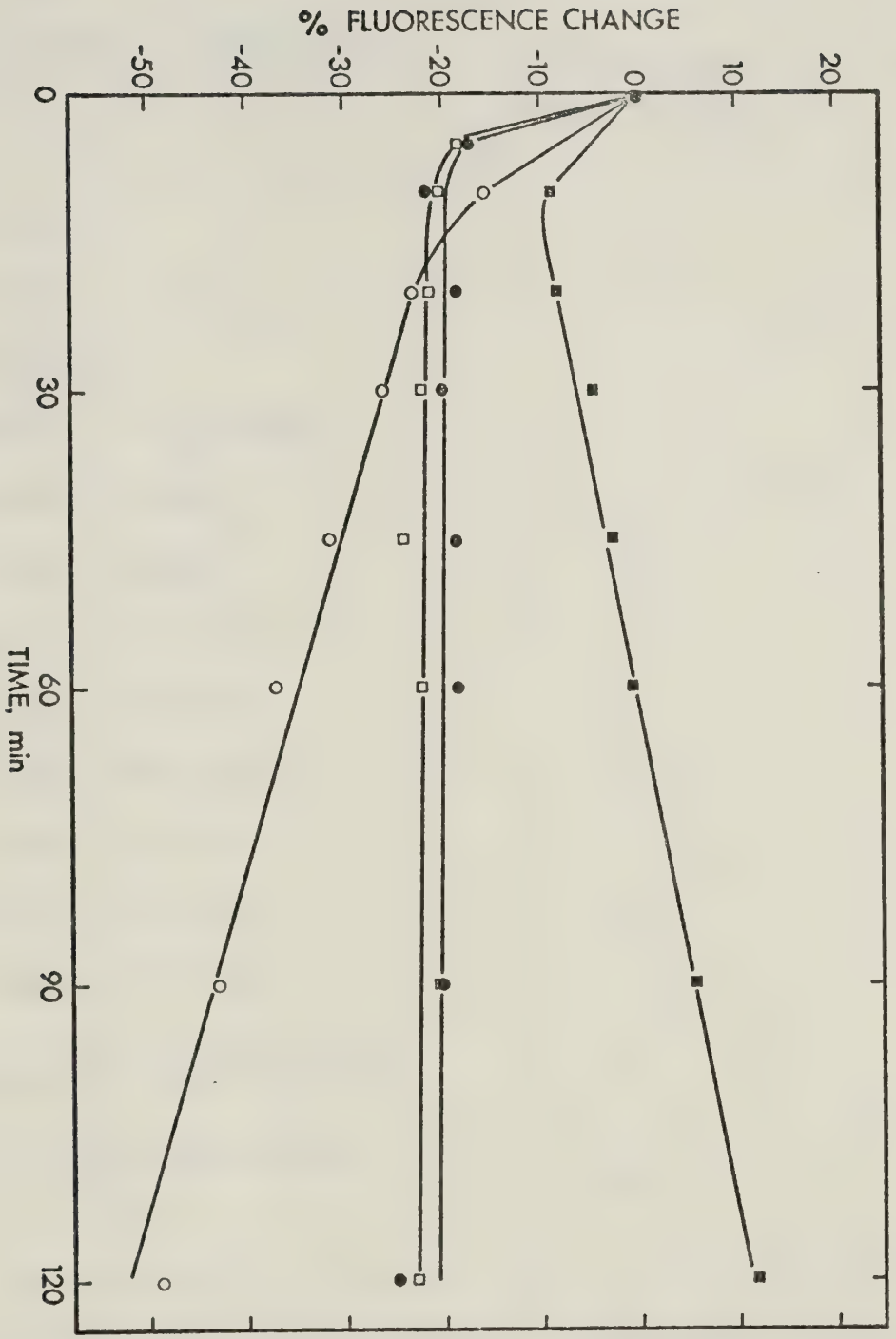

% FLUORESCENCE CHANGE
-50
-40
-30
-20
-10
0
10
20
0
30
60
90
120
TIME, min

TABLE 3.

Effect of Various Reaction Conditions on

E. coli sh DNA Relaxing Activity

Salts present in addition to 1 mM EDTA and 10 mM Tris, pH 8	% fluorescence drop before heat*	Relative fluorescence drop
2 mM $MgCl_2$	15	88
4 mM $MgCl_2$	17	100
20 mM $MgCl_2$	0	0
2 mM $MgCl_2$ + 6.7 mM EDTA	0	0
20 mM KCl, no $MgCl_2$	15	88
50 mM KCl, no $MgCl_2$	11	65
50 mM KCl, 20 mM EDTA	4	23
2 mM $MgCl_2$, 2% EtOH	14	82
2 mM $MgCl_2$, 20% EtOH	0	0
2 mM $MgCl_2$, 20 mM KCl	23	135
2 mM $MgCl_2$, 50 mM KCl	21	123
2 mM $MgCl_2$, 0.2 M KCl	0	0
0.01, 0.1 and 1 mM spermidine with 0, 0.1 and 0.2 M KCl	0	0
0.01 or 0.1 mM spermidine, 2 mM $MgCl_2$	20	117

* Essentially the same percentage of fluorescence drop was obtained after the heating cycle indicating no conversion of ccc-DNA to oc-DNA.

from 10 μM to 1 mM with KCl concentrations from 0 to 0.2 M in the absence of Mg^{++} resulted in no relaxation. On the other hand, in the presence of 2 mM Mg^{++}, the normal reaction concentration, a range of spermidine concentrations up to 100 μM was not inhibitory.

A brief study was conducted in which sh DNA relaxing reactions under standard conditions were run at pH's ranging from 4.5 to 10. The results indicated that a slightly alkaline pH, about 8, was preferred, with activity decreasing rapidly to the acidic side of pH 7.5. In standard 30 minute assays carried out at various temperature, maximal relaxation of PM2 DNA occurred in the range of 21° to 30°, with incomplete relaxation seen at all temperatures tested between 0° and 45°.

As indicated in Table 3, the maximum fluorescence drops generally observed with E. coli sh DNA relaxing protein was in the range of 15 to 20%, under standard reaction conditions. This was observed over a large number of assays with several preparations of the protein. If it is assumed that a 23% fluorescence drop indicates the maximum extent to which the supercoils in PM2 DNA can be released, and since a 33% fluorescence drop is indicative of complete relaxation, then the E. coli protein is capable of producing a 70% decrease in the superhelix density of the PM2 DNA, assuming that a linear relationship exists between the decrease in superhelix density and the fluorescence drop (Pulleyblank, 1974). These values are in reasonable agreement with the data of Wang (1971) who found that E. coli omega removed 110 of 150 (or 73%) superhelical turns from a species of λb2b5c DNA, using a sedimentation velocity-dye assay. In any case it is apparent that complete relaxation of negative supercoils was not achieved under any type of reaction conditions investigated.

Experiments designed to test for relaxation of positive supercoils all yielded negative results. In this feature as well, the results were in agreement with the data of Wang.

B. Assay for sh DNA Relaxing Activity in Small Colonies of *E. coli*

The gentle lysis procedure (de Lucia and Cairns, 1969) employed for obtaining relaxing protein from small colonies of *E. coli* yielded a crude cell free extract essentially free of DNA and containing minimal endonuclease activity. The lysate (total volume 160 µl) from four small colonies contained enough relaxing activity that 5 µl was sufficient to partially relax the DNA in a 50 µl standard reaction mixture. The drops in fluorescence enhancement in 30 minutes incubation at 30° were 10% before heating and 16% after the heat step. The larger loss in fluorescence after heating indicated some contaminating endonuclease activity, as explained above (see also Fig. 9). Since the procedure was intended primarily to provide a quick assay for the presence of relaxing activity and not as a method of quantitation, endonuclease at the levels encountered is not a serious problem.

C. *B. megaterium* sh DNA Relaxing Protein

The properties of *B. megaterium* sh DNA relaxing protein were found to be generally similar to those of the *E. coli* protein as reported here and by Wang (1971), and unlike the various eucaryotic relaxing proteins discussed in the Introduction (see Table 1). Table 4 summarizes the results with the *B. megaterium* protein. As with the *E. coli* protein, that from *B. megaterium* is dependent for activity on

TABLE 4.

Properties of sh DNA Relaxing Protein from B. megaterium

Reaction Conditions (All reactions contained 10 mM tris, pH 8, 2 mM $MgCl_2$ and 1 mM EDTA unless noted)	% fluorescence drop before heat	Relative fluorescence drop
standard reaction	26*	100
10 µM spermidine	27	104
100 µM spermidine	22	85
10 mM Na_3 EDTA	3	12
no $MgCl_2$	0	0
no $MgCl_2$ + 2 mM $CaCl_2$	26	100
no $MgCl_2$ + 2 mM $MnCl_2$	12	46
0.2 M NaCl	15†	58
0.4 M NaCl	0	0
EtOH (1% to 10%)	20-24	77-92
20% EtOH	0	0
naladixic acid, 1 mg/ml	0	0
20 µM pCMB	0	0
1 mM β-mercaptoethanol	22	85

All reactions were at 30° for 30 minutes.

* average of 8 determinations.

† average of 6 determinations.

the presence of divalent cation in the reaction mixture. Calcium appears to be as effective as Mg^{++}, but when 2 mM Mn^{++} was substituted, the fluorescence drop in 30 minutes was only about 50% as great as with Mg^{++} or Ca^{++}. Standard and kinetic fluorimetric assays showed that B. megaterium sh DNA relaxing protein was less sensitive to Na^{+} or K^{+} than the E. coli protein. For unknown reasons, agarose gel experiments did not confirm this finding. B. megaterium relaxing protein did not relax positively supercoiled DNA under any reaction conditions tested. Positively supercoiled DNA was generated by the addition of ethidium bromide to reaction mixtures containing PM2 DNA which had been relaxed with calf thymus omega protein (Pulleyblank and Morgan, 1975b). In this property also the B. megaterium protein resembled the E. coli protein. In a large number of 30 minute fluorescence assays using B. megaterium relaxing protein, the average before heat fluorescence drop observed was 26%, greater than the 15% to 20% normally observed under standard reaction conditions with the E. coli protein, but less than the 33% drop expected for complete relaxation. This drop is equivalent to release of about 87 of the 110 supercoils calculated to occur in native PM2 DNA. This should be regarded as a minimum value of the extent of relaxation, since the trace of endonuclease present in fraction IV would tend to lower the before heat fluorescence drop (see Fig. 6). Electron micrographs of native superhelical and relaxed PM2 DNA are shown in plates 1 and 2, respectively. Plate 3 illustrates an agarose gel electrophoresis experiment on the relaxing activity of fraction IV.

The activity of the B. megaterium protein was completely inhibited in the presence of pCMB at concentrations of 20 μM or greater

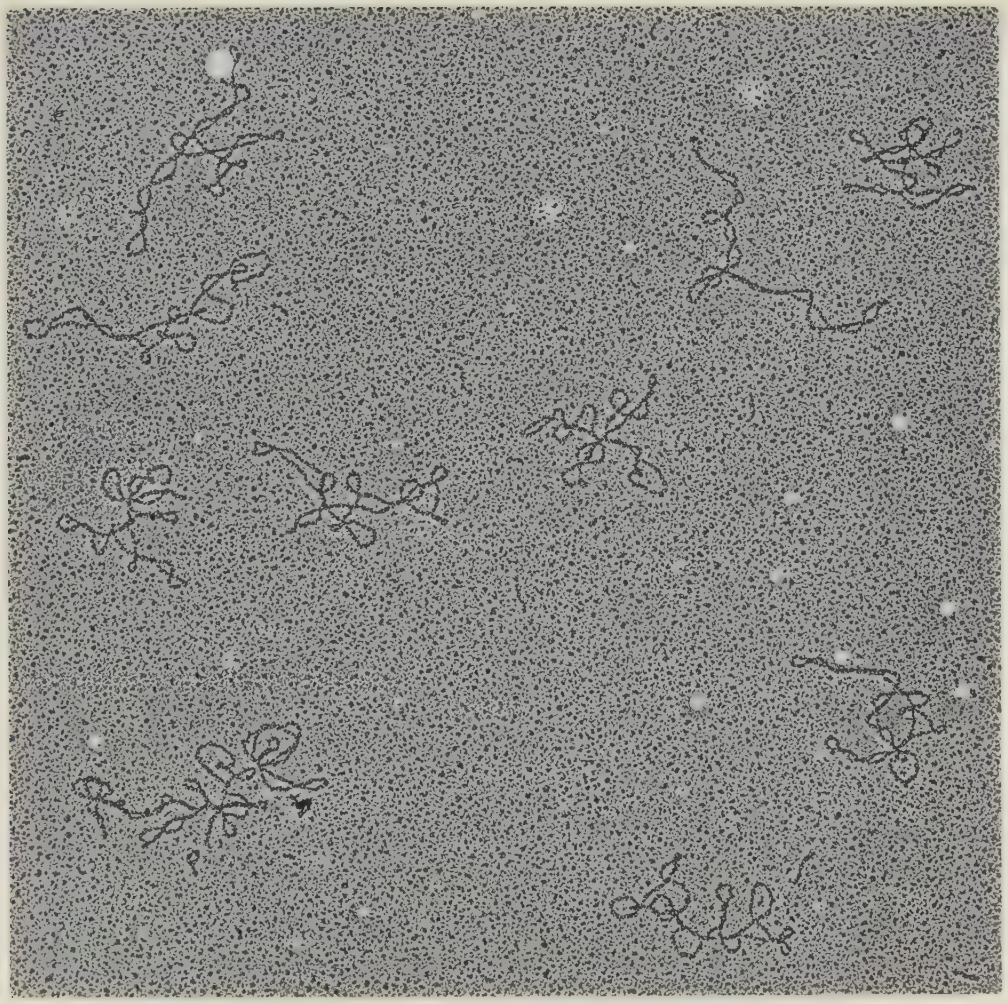


Plate 1 Native superhelical PM2 DNA. Magnification is 34,000X.

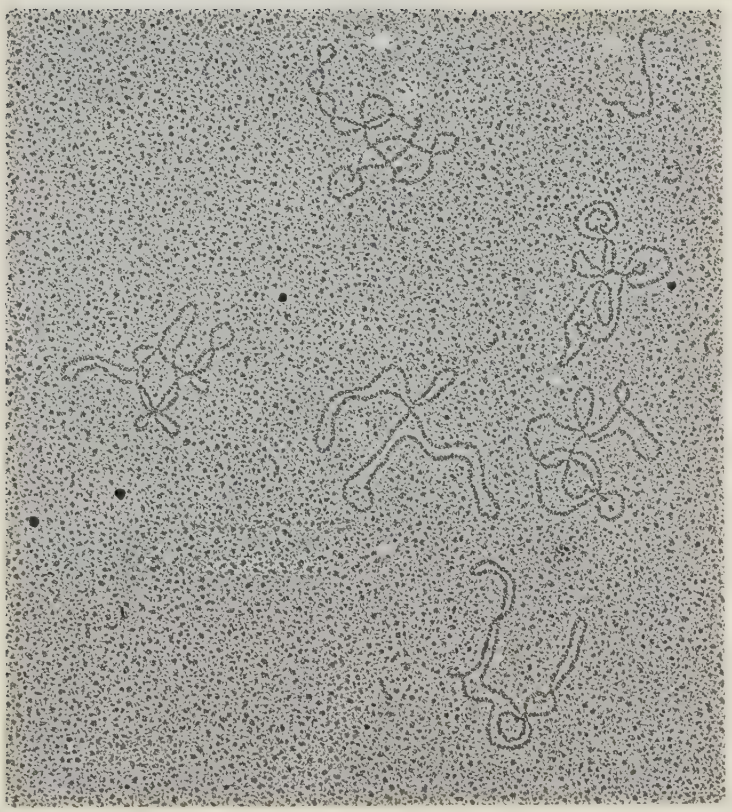


Plate 2 PM2 DNA incubated for 45 minutes with fraction IV *B. megaterium* sh DNA relaxing protein. Magnification is 34,000X.

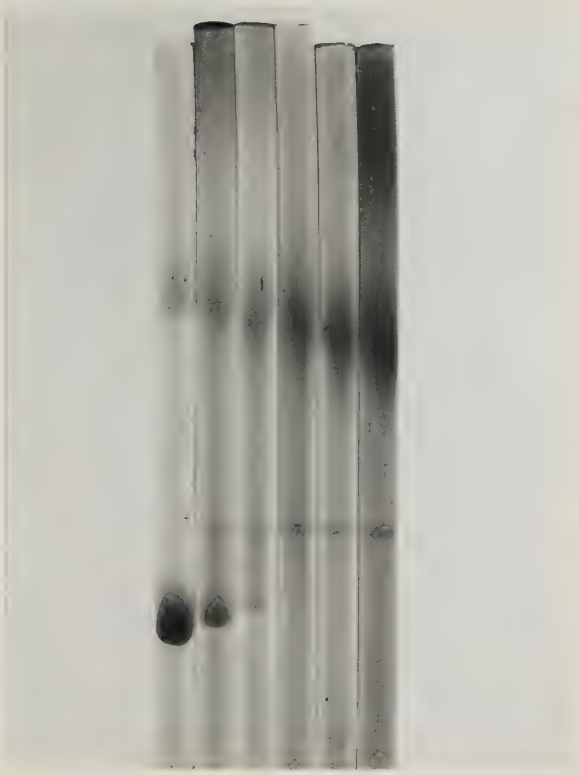


Plate 3 Agarose gel electrophoresis of PM2 DNA incubated with fraction IV B. megaterium sh DNA relaxing protein. Samples were removed after various incubation times and quenched with 0.2 M EDTA as described in the text. Incubation times were: gel no. 1, 0 min.; no. 2, 10 min.; no. 3, 20 min.; no. 4, 30 min.; no. 5, 45 min.; no. 6, 60 min. Direction of migration was from top to bottom, with the superhelical DNA migrating most rapidly.

or by naladixic acid (an inhibitor of replicative DNA synthesis of unknown mode of action) at 1 mg/ml. The fluorescence assays were confirmed by agarose gel electrophoresis (see plate 4).

Polyacrylamide-SDS gels of fraction IV protein showed a single major band (see plate 5). Comparison with E. coli RNA polymerase suggests a molecular weight of 120,000 for this band (Fig. 10). The low level of endonuclease present in fraction IV would not be expected to produce a visible band on a gel. No estimate of the extent of purification of the protein was possible since the activity was completely masked by endonuclease prior to the DNA agarose column. Fraction IV contained approximately 300 µg of protein, in the best preparation, from the 100 g of cells. Subsequent purifications were less successful, with barely detectable traces of relaxing activity being eluted from DNA agarose. No obvious reason for this problem has been found.

Fig. 11 shows some typical results of the treatment of fraction IV with trypsin. Fraction IV (100 µl) was incubated with 20 µl of trypsin (1 mg/ml) for 5 minutes; 15 µl of this solution was used in a kinetic assay for relaxing activity (lines "a" in Fig. 11). Also shown in the diagram are a typical sh DNA relaxation reaction with 10 µl of untreated fraction IV (b) and a control demonstrating the lack of endonuclease activity in the trypsin stock solution (c). The results suggested that preincubation of fraction IV with trypsin resulted in selective loss of relaxing activity and an increase in the endonuclease activity. One possible explanation for this observation would be that the trypsin treatment resulted in inactivation of the ligating portion of the nicking-closing activity of the sh DNA relaxing protein at a greater rate than that with which the endonucleolytic activity of the

Plate 4 Agarose gel electrophoresis of PM2 DNA incubated with fraction IV B. megaterium sh DNA relaxing protein under different reaction conditions. Each pair of gels represents the zero time (A) and 30 minute incubation (B) respectively. Reaction 1, standard reaction conditions; 2, +0.2 M NaCl; 3, +50 μM pCMB; 4, standard reaction conditions; 5, + naladixic acid, 1 mg/ml; 6, calf thymus omega protein reaction.

Plate 5 SDS-polyacrylamide gel electrophoresis of fraction IV *B. megaterium* sh DNA relaxing protein.

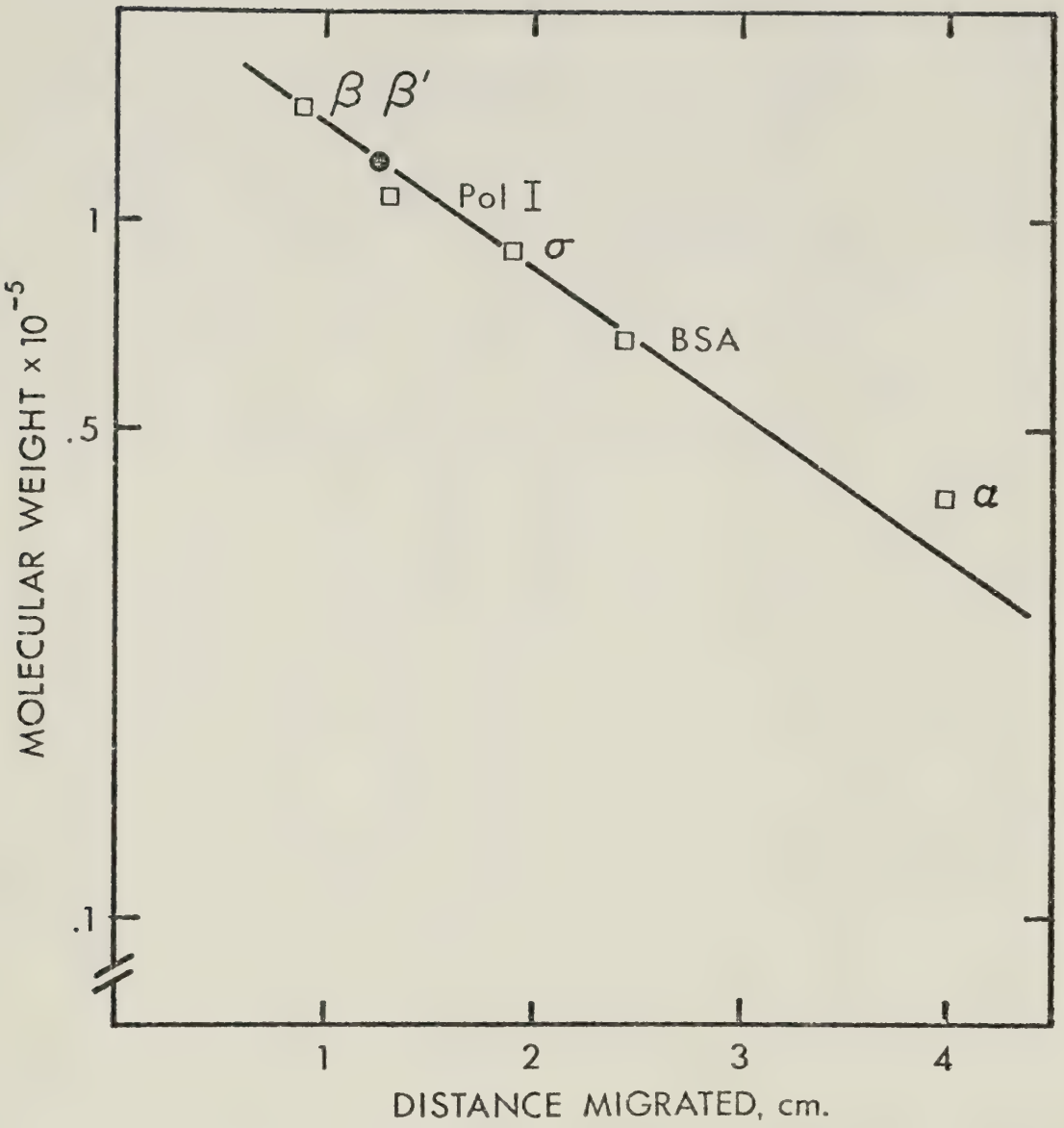


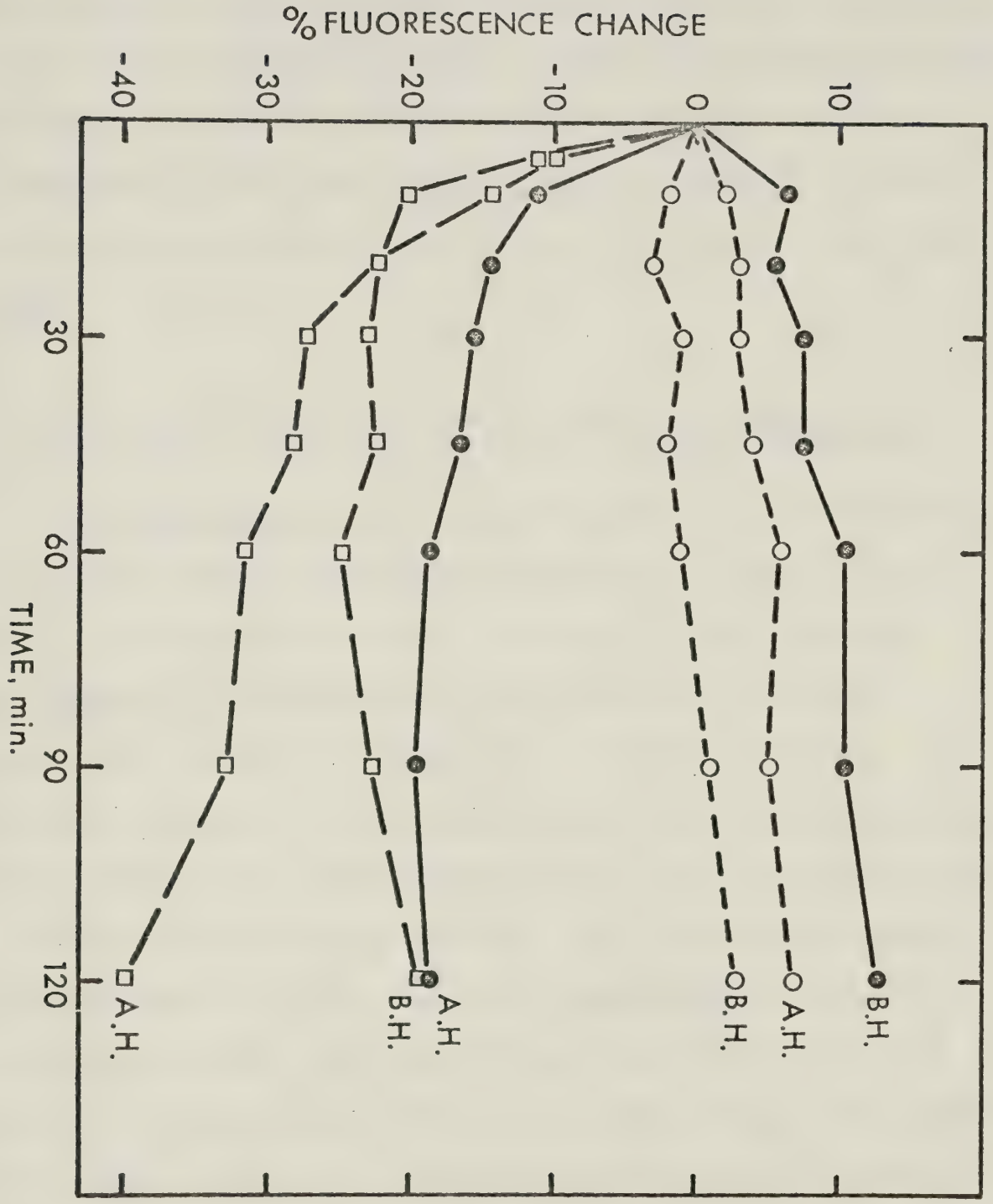
Figure 10 Molecular weight determination for B. megaterium sh DNA relaxing protein (O) with E. coli RNA polymerase (β, β', α, σ) polymerase I and BSA as molecular weight standards.

Figure 11 Effect of limited proteolytic treatment on the activity of B. megaterium sh DNA relaxing protein. All reactions were 150 µl, from which 15 µl was removed at each time point for fluorescence assay.

a). Fraction IV treated with trypsin (166 µg/ml) for 5 minutes at 22°; 15 µl of trypsin digested, fraction IV used in the reaction mixture (i.e. equivalent to 12.5 µl of fraction IV and 2.5 µl trypsin). ●——●

b). 10 µl of normal fraction IV in the 150 µl reaction. □— —□

c). 10 µl of stock solution of trypsin (1 mg/ml) in the 150 µl reaction. 0- - -0.


% FLUORESCENCE CHANGE
-40
-30
-20
-10
0
10
TIME, min.
30
60
90
120
A.H.
B.H.
A.H.
B.H.
B.H.
A.H.
B.H.
A.H.

protein was degraded. If this were the case, sh DNA relaxing protein would be converted to an endonuclease. Such a result would seem to imply that there are two separate regions in the active site, and that the ligating region is more susceptible to trypsin digestion. The presence of a trace of endonuclease in fraction IV means that the trypsin experiments must be interpreted with caution, since it is not impossible that the results could be due to the removal by proteolytic digestion of some factor which inhibits the activity of the contaminating endonuclease.

D. sh DNA Relaxing Activity in a Crude Lysate of M. lysodeikticus

M. lysodeikticus proved to be a rich source of sh DNA relaxing protein, with activity readily detectable even in a crude cell-free extract. The protein concentration in fraction II was 0.8 mg/ml. Table 5 summarizes the properties of the activity in the crude fraction. The low level of endonuclease in the high salt wash (fraction II) suggested that M. lysodeikticus rather than B. megaterium should be considered as the organism of choice for routine preparation of procaryotic sh DNA relaxing protein. Fig. 12 illustrates kinetic reactions carried out at two levels of M. lysodeikticus protein; the near equivalence of the fluorescence drops before and after heat is indicative of very low levels of detectable endonuclease. The presence of the relaxing activity was confirmed by agarose gel electrophoresis. As was the case with other procaryotic sh DNA relaxing proteins, the M. lysodeikticus activity required divalent cations with Ca^{++} being nearly as effective as Mg^{++}. The addition of 0.2 M NaCl did not

Figure 12 Kinetics of relaxation of PM2 ccc DNA by crude M. lysodiekticus sh DNA relaxing protein. Reactions were under standard conditions in a total volume of 150 µl at a PM2 DNA concentration of 1.2 A_{260}/ml. Aliquots of 15 µl were removed at each time point for fluorescence assay.

a). 5 µl of crude fraction ■—■

b). 30 µl of crude fraction O---O

Fluorescence readings are given in arbitrary units.

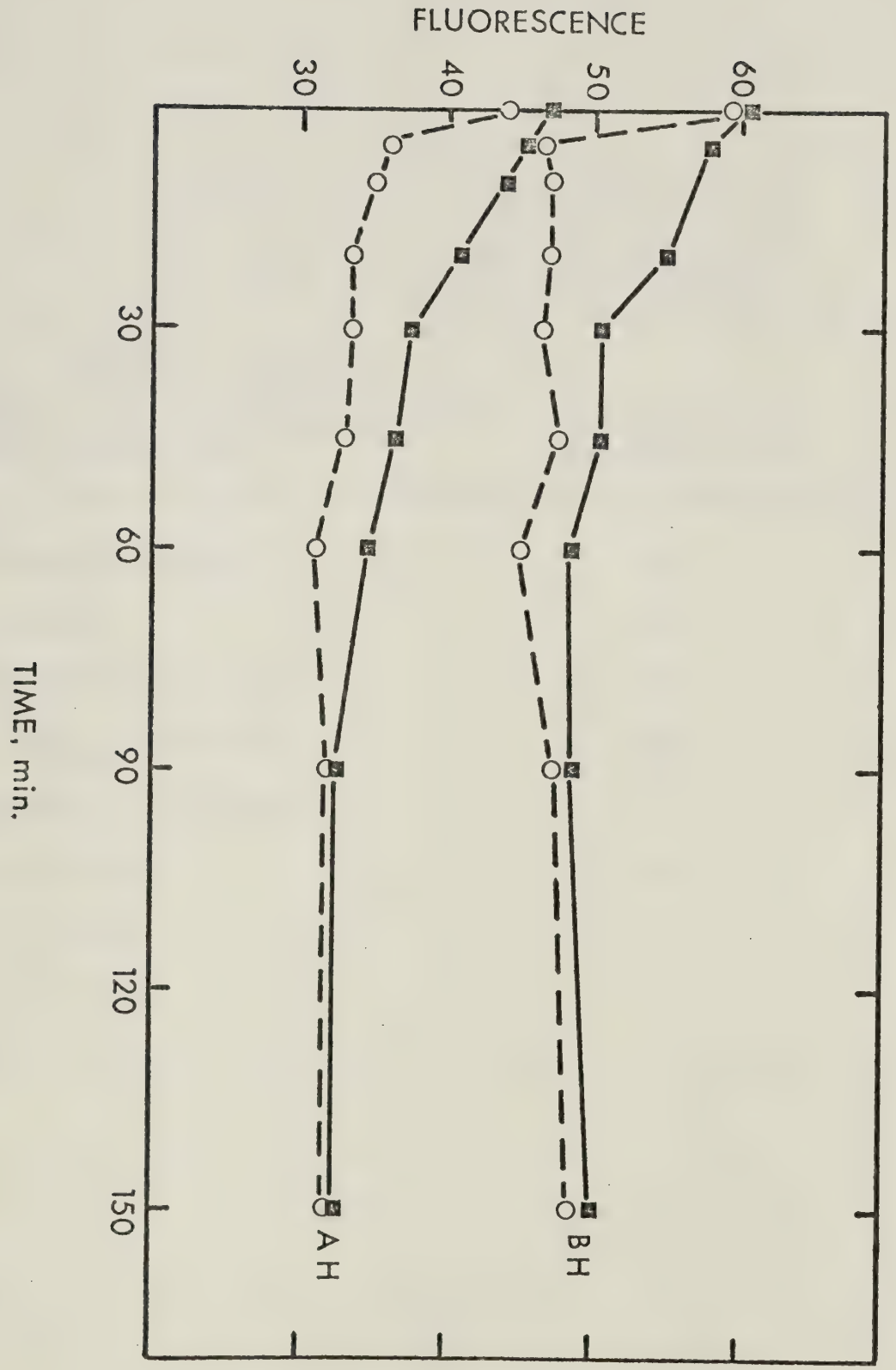

FLUORESCENCE
30
40
50
60
30
60
90
120
150
TIME, min.
A H
B H

TABLE 5.

Properties of the sh DNA Relaxing Activity

in a Crude Extract from M. lysodeikticus

Reaction Conditions (All reactions contained 10 mM tris, pH 8, 2 mM $MgCl_2$ and 1 mM EDTA unless noted)	% fluorescence drop before heat	Relative fluorescence drop
standard reaction	28	100
no $MgCl_2$	0	0
no $MgCl_2$ + 2 mM $CaCl_2$	20	72
no $MgCl_2$ + 5 mM $CaCl_2$	23	82
no $MgCl_2$ + 0.2 M NaCl	6	21
0.2 M NaCl	27	96
0.4 M NaCl	7	25
0.5 mM pCMB	0	0

inhibit the activity if Mg^{++} was also present; higher concentrations (0.3 M or more) reduced the activity to low levels. No relaxation of a positively supercoiled DNA substrate was observed under conditions similar to those tested for the E. coli and B. megaterium proteins. Since the active fraction (II) used was relatively crude, the failures of the more purified proteins to use positively supertwisted DNA as a substrate would appear less likely to be a consequence of the removal during purification of a critical factor which is present in vivo. Polyacrylamide-SDS gel electrophoresis of fraction II resolved six major and several minor bands, therefore, no estimation of molecular weight or subunit composition was possible (the size range of the major bands was from approximately 100,000 to 40,000 daltons). Activity progressively decreased upon the addition to the reaction of increasing concentrations of pCMB, but was not completely abolished at concentrations less than 0.5 mM. The activity appeared generally very similar to the other procaryotic proteins studied with the exception of the apparently decreased sensitivity to monovalent cation.

CHAPTER IV

DISCUSSION

The results reported here, together with the studies of Wang (1971b, 1973) suggest that a fundamental difference may exist between the properties of procaryotic and eucaryotic sh DNA relaxing proteins, this being the apparent selectivity of the procaryotic proteins for negatively supercoiled DNA. It cannot be excluded, however, that the observations in this regard represent an artifact of the purification or assay procedures employed. It may be, for instance, that the presence of intercalated or free ethidium bromide in the reaction mixtures for positive supercoil relaxation inhibits the binding or activity of the enzyme. It has been shown (Richardson, 1973; Richardson and Parker, 1973) that ethidium can inhibit the ability of E. coli RNA polymerase to transcribe a circular or linear DNA template. In our own group it was found that histone H1 is able to compete with ethidium for binding sites on calf thymus DNA, resulting in abolition of fluorescence enhancement. This observation formed the basis of the assay for proteolytic enzyme activity described earlier. These two examples indicate that dye and protein binding to DNA can at least under certain conditions be mutually antagonistic; and it is therefore possible that the present observations are due to a similar competition. This possibility will only be resolved if a method is found whereby a DNA of fairly high positive superhelix density can be generated in the absence of ethidium bromide.

The unwinding of duplex DNA which must accompany replication

would be expected to give rise to positive supercoils in a ccc duplex in the region ahead of the replication fork (Fig. 1B). Since these supertwists must be released if replication is to continue, an obvious role is suggested for sh DNA relaxing proteins capable of relaxing positive supercoils. The recent isolation of a sh DNA relaxing activity from rat liver mitochondria (Fairfield et al., 1976) is particularly gratifying in this regard, given the ccc duplex structure of mt DNA. It seems reasonable to assume that some mechanism must also be available to the bacterial cell for the relaxation of positive supercoils. The proteins studied in this work, may, of course, be specific for negative supercoils in vivo, in which case an as yet undiscovered protein could be responsible for relaxation of positive supercoils. Such an activity could easily be masked in crude extracts which contain high levels of endonucleolytic activity. Failure of the crude lysate of M. lysodeikticus, which was very low in endonuclease, to relax positively supercoiled PM2 DNA would tend to argue against this possibility, however. A third alternative which should be considered is that positive relaxation could be carried out in vivo by a concerted reaction of a known endonuclease and polynucleotide ligase and that searches for a specific positive sh DNA relaxing enzyme will continue to prove fruitless. But the same pair of enzymes should be equally capable of relaxing negative supercoils, yet proteins whose specific function is to relax these have been demonstrated to exist in every type of organism which anyone has bothered to test. A further explanation could be that the putative positive relaxation activity exists as an uncharacterized function of a known enzyme. The data of Champoux and McConaughy (1975) showed that replication of SV40 DNA in vitro was accompanied by a slow

rate of template nicking although their DNA polymerase was free of conventional endonucleolytic activity. The suggestion was advanced that the polymerase was recognizing some abnormal structural feature of the template which resulted from the presence of positive supercoils and was capable of creating a nick at such a site.

Proteins specific for negative supercoils are unlikely to function in replication according to most model predictions. One recent model (Morgan, 1970), however, involving covalent linkage of the daughter strands, could result in negative supercoiling behind the replication fork. Involvement of these proteins in transcriptional control appears reasonable. Worcel and Burgi (1972, 1974) have shown that the E. coli nucleoid consists of between 12 and 80 topologically independent negatively supercoiled loops, radiating from a central core to which the loops are fixed by RNA. As discussed in the Introduction, a large body of evidence has accumulated indicating that the ability of E. coli RNA polymerase to transcribe a circular DNA template is highly sensitive to the degree of supercoiling of that template. Although direct evidence is still lacking, it is apparent that sh DNA relaxing proteins could be capable of regulating the rate of transcription of a particular segment of DNA by altering the superhelix density. Since the reduction in number of superhelical turns appears to proceed one turn at a time, a degree of "fine-tuning" of the superhelix density of the DNA segment, and thus of its rate of transcription, is theoretically possible. The difficulty with this proposal is that a mechanism for re-winding the DNA to its original superhelix density is lacking; the superhelix relaxing reaction is as far as is known essentially irreversible, except for small fluctuations around $\tau = 0$ (Pulleyblank et al., 1975).

Recombination may also be subject to regulation by sh DNA relaxing proteins, since Holloman et al. (1975) have shown that superhelix density influences a process thought to be involved in the initiation of recombination.

At the present time, no mutants defective in sh DNA relaxing activity have been reported from either a procaryotic or eucaryotic source. Until such mutants can be isolated and characterized the in vivo function of these proteins will probably remain unclear. As was shown in the Results, it is possible to detect superhelix relaxing activity in small colonies of E. coli employing a rapid, simple lysis procedure. This technique could be of use in a system designed to screen large numbers of colonies for activity - a reasonable estimate would be that 100 could be assayed per day.

Some of the properties of the three procaryotic sh DNA relaxing proteins studied are summarized in Table 6. As has already been discussed, the three share the characteristic of apparent specificity for negatively supercoiled DNA. All are dependent on the presence of Mg^{++} in the reaction mixture, none was active when Mg^{++} was replaced by 0.2 M NaCl, conditions generally near the optimum for the eucaryotic sh DNA relaxing proteins (see Table 1). Sensitivity to NaCl in the presence of Mg^{++} was variable, with the E. coli protein being completely inhibited and that of M. lysodeikticus retaining over 50% of its maximum activity. There was a large variation in the level of pCMB required to inhibit the activity of the procaryotic proteins, with B. megaterium being the most sensitive; activity was completely inhibited at concentrations of 20 μM or higher, indicating the existence of an essential sulfhydryl group. This level was less than 1/10 the concentration

TABLE 6.

Comparison of Procaryotic sh DNA Relaxing Proteins

	E. coli	*B. megaterium*	*M. lysodeikticus*
maximum % fluorescence drop under standard conditions*	17	26	28
% relaxation (for 33% fluorescence drop ≡ 100% relaxation)	52	79	85
% fluorescence drop in presence of 0.2 M NaCl (+ 2 mM Mg^{++})	0	15	21
level of pCMB required to reduce activity to 0	1 mM +	20 μM	0.5 mM
relaxation of positive supercoils	no	no	no
requirement for Mg^{++}	yes	yes	yes
molecular weight	87,000	~ 120,000	?

* Standard conditions are 10 mM tris, pH 8, 2 mM Mg^{++}, 1 mM Na_3EDTA, 30 minute incubation at 30°.

required to inhibit the E. coli and M. lysodeikticus proteins. pCMB has been shown to inhibit eucaryotic sh DNA relaxing proteins isolated from human KB (Keller, 1975a) and mouse LA9 cells (Vosberg and Vinograd, 1974).

A possibly important difference observed among the three procaryotic proteins was in the extent of relaxation of supercoils, as measured by the percent fluorescence drop in 30-minute and kinetic experiments. Where the E. coli protein generally produced a maximum fluorescence drop in the range of 15% to 20% under standard assay conditions, the B. megaterium protein and the crude M. lysodeikticus extract gave reductions of between 25% and 30%, much closer to the 33% drop associated with complete relaxation. In the case of M. lysodeikticus the possibility of a stimulatory factor being present in the crude fraction cannot be ruled out; this seems very unlikely, however, to be true of the B. megaterium protein, which was substantially purified (plate 5). Direct resolution of this problem in the E. coli situation was not possible because the endonuclease contamination of impure fractions masked the activity of the superhelix relaxing protein. In any event, none of the procaryotic proteins was able to catalyze complete relaxation of PM2 DNA, another major point of distinction from the eucaryotic sh DNA relaxing proteins. Wang (1971b) attributed this property to a progressive reduction in the driving torque of the reaction as the superhelix density of the DNA substrate decreased, basing this explanation on the observed decrease in reaction rate with time of incubation. The results of kinetic fluorescence experiments reported here (Fig. 9) support this interpretation.

The only significant point of difference between the data

reported here for the E. coli sh DNA relaxing protein and in the papers by Wang (1971b, 1973) for E. coli omega protein is the molecular weight. One explanation for this observation would be that the 56,000 and 31,000 dalton subunits which have been observed are produced by proteolytic digestion of the native protein (molecular weight 110,000). This seems unlikely on two counts. First, the two subunits together form a structure of less than 90,000 daltons, meaning that an approximately 20,000 dalton section of the molecule would have to be lost altogether without seriously impairing the catalytic activity of the protein. Secondly, if proteolytic cleavage was taking place, it seems reasonable to expect that some intact protein would be seen on the SDS-polyacrylamide gels, yet the gels for fractions V and VI showed only the two bands seen in the scan (Fig. 7A). That proteolysis cannot be completely discounted, however, is shown by the example of E. coli DNA polymerase I, which can be processed into two fragments in crude extracts (Setlow et al., 1972).

A second possible explanation which must be considered in this regard is that different strains of E. coli were used as enzyme sources. The experiments reported here made use of protein isolated from E. coli B, while the reports of Wang (1971b, 1973) concern the protein found in E. coli 1100; therefore, the difference in molecular weight and subunit structure may be a consequence of the choice of organism.

The finding of two subunits in the protein was particularly intriguing since the catalytic activity is bifunctional and it would be interesting to attempt to separate the subunits and assign a specific role to each. Some preliminary experiments involving urea denaturation of the purified protein were attempted, but no activity could be

recovered upon removal of urea by dialysis. Further experiments in this area were not attempted due to the relatively poor storage stability of the purified fraction.

Two eucaryotic sh DNA relaxing proteins, isolated from mouse cells (Vosberg and Vinograd, 1974) and from human KB cells (Keller, 1975c) are believed to consist of two subunits, although in both cases the subunits are apparently of identical molecular weight.

The experiments where B. megaterium sh DNA relaxing protein was subjected to limited trypsin digestion are, unfortunately, not as clear as would be desirable. It appeared that the short, low temperature trypsin treatment employed was selectively inactivating the ligating portion of the nicking-closing activity, while leaving the endonucleolytic function relatively intact. Since fraction IV (the most purified fraction of this protein) contained trace amounts of endonuclease which could be detected during kinetic relaxation experiments of several hours' duration (Fig. 6), the effect of trypsin on fraction IV activity must be interpreted with some caution. It could be argued, for example, that the effect of trypsin is to totally inactivate sh DNA relaxing activity in fraction IV, rendering the contaminating endonuclease more readily assayable. It was seen from some of the time-course experiments, however, that the rate of nicking was considerably greater for trypsin-treated fraction IV protein than for a similar quantity of the "native" protein (Fig. 11), suggesting an increase in the occurrence of unsealed nicks. A control experiment showed that no endonuclease activity was associated with the trypsin itself.

If, on the other hand, the results are accepted at face value they suggest that, since the closing portion of the nicking-closing

activity is preferentially inactivated, different regions of the polypeptide chain may be involved in carrying out the two functions. This selective inactivation could be useful in attempting to isolate and characterize the nicked DNA-protein intermediate in the relaxation reaction.

The sensitivity of the B. megaterium sh DNA relaxing protein to inhibition by naladixic acid is potentially, at least, one of the most interesting aspects of these studies. Naladixic acid is an inhibitor of bacterial replicative DNA synthesis whose exact mechanism of action has not been discovered. These experiments at least suggest that the relaxing protein may play a role in replication, despite its apparent inability to relax positively supercoiled DNA.

The full potential of the agarose gel electrophoresis method has not been exploited in the experiments reported here. A difficulty encountered in the use of tube agarose gels was the non-uniform shrinkage of some gels during electrophoresis. Within a single set of 12 gels, shrinkage often ranged from zero to upwards of 3 cm from an initial length of 14 cm. Although the gels tended to regain at least some of the lost length when soaked in staining solution or water, direct comparison of distances migrated was of necessity somewhat uncertain. For this reason the gel assay was employed only in a diagnostic fashion (i.e. to confirm the fluorimetric assays for sh DNA relaxation) and not as a method of quantitating the extent of relaxation, as had been reported in the literature (see Chapter I). The most reasonable solution to the problem would be the use of slab gels, where if shrinkage occurred, it would be expected to be constant

across the whole gel.

The original aim of this work was to answer certain questions regarding the properties of E. coli sh DNA relaxing (omega) protein and its relationship to a variety of apparently similar activities found in eucaryotic cells. The results have shown that the E. coli protein is not unique; sh DNA relaxing proteins from the procaryotic species appear to share the characteristics which marked the E. coli protein as being distinct from the mouse embryo, Drosophila egg and other eucaryotic proteins. What this may mean in terms of mechanisms of DNA replication and regulation of gene expression remains to be seen, although it is possible to indulge in a good deal of speculation. Certainly the data presented ask as many questions as are answered. As has been stressed repeatedly, the crucial work in this area will be the phenotypic characterization of a mutant defective in sh DNA relaxing activity. Other aspects of this work on which further study could be rewarding include the subunit composition of the E. coli protein, the selective inactivation of the B. megaterium closing function and the meaning of the naladixic acid inhibition data with reference to the in vivo function of the protein.

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APPENDIX

Calculation of Units of sh DNA Relaxing Activity

A unit of activity is defined as that amount of protein which causes a 15% drop in the BH fluorescence of 1 µg of RFI PM2 DNA in 30 minutes at 30° under the standard assay conditions. This is calculated from the measured fluorescence drop between zero and 30 minutes as a fraction of the total fluorescence due to renaturing DNA in the zero time sample from the reaction under consideration:

$$\% \text{ fluor. drop} = \frac{\Delta BH}{AH_{t=o}} \times 100 \qquad (1\text{-A})$$

where ΔBH is the fluorescence drop in arbitrary units, and $AH_{t=o}$ is the fluorescence yield of the ccc DNA in the reaction.

The number of units in a protein fraction, then, is:

$$\text{No. units} = \frac{\% \text{ fluor. drop}}{15\%} \times \mu g \text{ ccc DNA} \times \frac{30 \text{ min}}{\text{reaction time}} \times \frac{1}{\text{dilution factor (if any)}} \qquad (2\text{-A})$$

Since the stock PM2 DNA solution is not 100% ccc, a correction must be applied to exclude the oc DNA from the calculation. This is done by comparing the BH and AH fluorescence readings from a control reaction performed routinely with each series of assays:

$$\text{fraction ccc DNA} = \frac{AH}{BH} \qquad (3\text{-A})$$

For a standard reaction, i.e., 50 µl containing PM2 DNA at $1A_{260}$ (50 µg/ml)

$$\mu g \text{ ccc DNA} = 50 \mu g/ml \times 0.05 \text{ ml} \times \frac{AH}{BH}$$

$$= 2.5 \mu g \times \frac{AH}{BH} \qquad (4\text{-A})$$

$$\text{units} = \frac{\% \text{ fluor. drop}}{15\%} \times 2.5 \mu g \times \frac{AH}{BH} \times \frac{30 \text{ min}}{\text{reaction time}} \times \frac{1}{\text{dilution factor}} \qquad (5\text{-A})$$

This formulation is adequate only for fractions which are free of endonuclease, which interferes with the fluorescence assay for sh DNA relaxing protein. For endonucleolytic activity on sh DNA, nicking of all molecules at least once results in a 30% fluorescence increase BH and

a corresponding 100% decrease AH (Morgan and Pulleyblank, 1974). Therefore, contamination of sh DNA relaxing activity with nuclease will result in a diminished fluorescence drop before heating (ΔBH) and an increased drop after heating (ΔAH). A correction for this must be made before calculation of units of activity can be performed:

$$\Delta BH_{true}\text{(due to sh DNA relaxation)} = \Delta BH_{meas.} + 0.3(\Delta AH_{meas.} - \Delta BH_{meas.}) \qquad (6\text{-A})$$

$$\%\ \text{fluor. drop} = \frac{\Delta BH_{true}}{AH_{t=o}} \times 100 = \frac{\Delta BH_{meas.} + 0.3(\Delta AH_{meas.} - \Delta BH_{meas})}{AH_{t=0}} \times 100 \qquad (7\text{-A})$$

This value is then substituted in eq. (5-A):

$$\text{units} = \frac{\dfrac{\Delta BH_{meas.} + 0.3(\Delta AH_{meas.} - \Delta BH_{meas.})}{AH_{t=0}} \times 100}{15\%} \times$$

$$2.5\ \mu g \times \frac{AH}{BH} \times \frac{30\ \text{min.}}{\text{reaction time}} \times \text{dilution factor} \qquad (8\text{-A})$$